Title

Epigenetic effects of environmental stressors

By

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Acknowledgments

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Index

Abbreviations

C5	Carbon 5
5hmC	5-hydroxymethylcytosine
5mC	DNA methylation
APC	Adenomatous polyposis coli
Atm	Ataxia telangiectasia mutated
bp	Base pairs
C	Cytosines
CDH1	Cadherin 1
CDKN2A	CDKN2A cyclin-dependent kinase inhibitor 2A
CDKN2B	CDKN2A cyclin-dependent kinase inhibitor 2B
CGIs	CpG islands
Ch3	Methyl
CTE	Chronic toxic encephalopathy
DAPK1	Death associated protein kinase 1
DNMTs	DNA methyltransferases
G	Guanine
Gpx	Glutathione peroxidase
Gsr	Glutathione reductase
GSTP1	GlutathioneS-transferase pi 1
HCC	Hepatocellular carcinoma
HDACs	Histone deacetylases
K-ras	Kirsten rat sarcoma
LC-MS/MS	Liquid chromatography–tandem mass spectrometry

MBD	Methyl-DNA binding domain
MBD1	Methyl-CpG-binding domain protein 1
MeCP2	Methyl CpG binding protein 2
microRNA	miRNA
nt	Nucleotides
PAH	Polyaromatic hydrocarbons
PKC	Protein kinase C
PM	Particulate matter
Pparg	Peroxisome proliferator-activated receptor gamma
PTMs	Post-transcriptional modification
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
Sin3a	Paired amphipathic helix protein
Tet	Ten-eleven translocation methylcytosine dioxygenase
TK6	Thymidine kinase
TRD	Transcription-regulatory domain
Trp53	Tumor protein p53
TSS	Transcription start sites
VHL	Von Hippel–Lindau tumor suppressor

1. Chapter 1

1.1.Introduction

Humans are exposed to a variety of environmental stressors e.g., chemicals, solvents, pollutants, lifestyle factors and microorganisms, during their life span (figure 1). Over the past few decades, scientific research has linked exposure of environmental stressors to human diseases (Ziech et al., 2010b). Human exposure to environmental agents causes more than 13 million deaths annually, and 24% of the diseases are estimated to be caused by environmental stressors worldwide (Hou et al., 2012). Exposure to environmental stressors is linked with several multifactorial diseases e.g., cancer, cardiovascular, autoimmune disease etc (Miller et al., 2012, Joseph et al., 2013). It has been estimated that 10% of about 80.000 chemicals in use today are recognized carcinogens (Carpenter et al., 2002). Higginson and Muir in 1977 asserted that 80% of all cancers were partly due to environmental exposures (Higginson and Muir, 1977). More recent estimations of environmental factors contributing to cancer, however, range from 1 to 19 % (Wild, 2009). It has thus of great interest not only to identify environmental stressors that can induce carcinogenesis (i.e., cellular and molecular events leading cancer), but also to elucidate the mechanisms by which they exert their carcinogenic effect. Traditional concept of chemical induced carcinogenesis is mainly based on mutational events leading to changes in the DNA sequence i.e., genetic event. Novel knowledge, however, also linked the occurrence of non-mutational events which regulate genes activity i.e., epigenetic events, in the aetiology of chemical carcinogenesis (Lund, 2011).

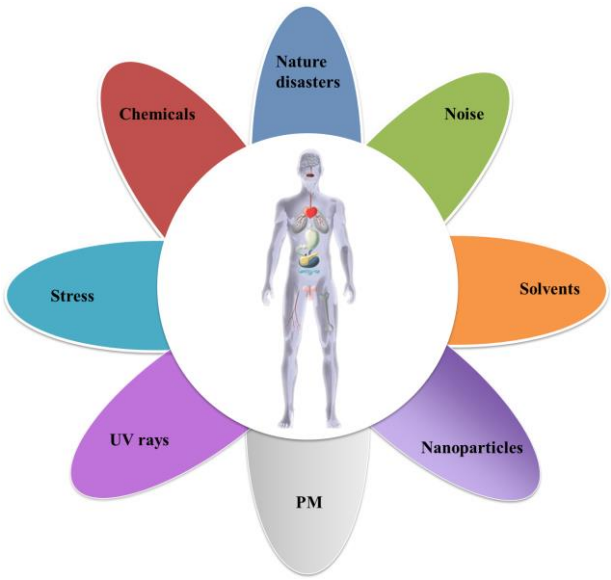

Figure 1: Human exposure to environmental stressors.

Humans are exposed different environmental stressor during lifetime as shown in figure. PM: particulate matter; UV: ultra violet.

1.2. Environmental carcinogenesis

Early experiments in understanding the process of carcinogenesis were done in mice skin exposed to coal tar that lead to the formation of skin tumour. The molecular changes in the mouse skins were explained by the multistage model of carcinogenesis (Abel and DiGiovanni, 2011). This model described that carcinogenesis initiates and proceeds through changes in the DNA (i.e., genetic effects) (Vogelstein and Kinzler, 2004). Molecular events encompassing multistep model of carcinogenesis comprise three phases i.e., initiation phase, promotion phase and progression phase. Initiation phase of carcinogenesis is characterized by the induction of irreversible DNA damage (e.g., mutation via DNA depurination and deamination) which pre-disposes cells to malignancy. Initiated cells are phenotypically similar to remaining cells but they have accumulated genetic damage, which may lead to high proliferation rate with less time for DNA repair. Initiation is irreversible genetic event, which is transmitted during mitosis. Promotion phase contributes to carcinogenesis by increased mitogenesis and by fixing mutations, although additional genotoxic events are not necessary. Both initiation and promotion phases of carcinogenesis are dependent on the presence of carcinogens in cells. Progression is the last phase of carcinogenesis during which cells acquire neoplastic phenotype. In this phase, cell proliferation is independent of the presence of carcinogens in cells, and is characterized by irreversibility, genetic instability, faster growth, invasion and metastasis of neoplastic cells (Oliveira et al., 2007).

1.2.1. Genetic mechanisms in carcinogenesis

The “initiation phase” of multistage carcinogenesis in mouse skin occurred with the dermal application of a genotoxic chemical e.g., benzo(a)pyrene. The initiated cells harbouring the genetic aberrations (i.e., DNA mutation) were reported after 1 week of dermal exposure of genotoxic chemicals. Mutations in *Hras1* gene lead to the clonal origin of the papillomas. In the initiation phase of carcinogenesis, chemical exposure

can induce many DNA lesions e.g., DNA adducts formation, DNA cross-links, DNA single or double strand breaks, gene copy number alterations. These DNA lesions could alter the expression and functions of genes, consequently leading to carcinogenesis (Abel and DiGiovanni, 2015).

In contrast to tumour initiators, tumour promoters exert their effects through altering the cell receptors, differentiation and signalling pathways. Tumour promoters do not directly affect the DNA, but rather induce their effect via reversible non-genetic events. In the mouse model of carcinogenesis, alterations in the signalling pathway of protein kinase C (PKC) were frequently implicated in the expansion of initiated cells.

The progression stage of carcinogenesis in the mouse model was accompanied by the occurrence of additional genetic events e.g., aneuploidy. In support of genetic involvement in multistage carcinogenesis model, DNA aberrations in the different cancers have been reported. For example, mutation in adenomatous polyposis coli (*APC*) gene, *K-RAS* and mutations in other oncogenes and tumor suppressor genes are described to take place in colorectal cancer. Similarly, mutations in oncogenes and tumour suppressor genes are also described in the carcinogenesis of squamous-cell lung carcinoma, osteocarcinoma, melanoma, retinoblastoma (Sadikovic et al., 2008).

Many chemicals present in the environment are characterized for their genotoxic potential. Hence exposure to these environmental chemicals could potentially lead to the development of cancer cells. Exposure to volatile compounds (benzene, hydroquinone etc) is shown to induce DNA damage (Harvilchuck et al., 2009). Polyaromatic hydrocarbons (PAH) e.g., benzo[a]pyrene exposure induces DNA adduct formation with purine bases which leads to genetic damage (Phillips, 1983). Also, exposure to halogenated compounds e.g., tricholoroethylene, carbontetrachloride are shown to induce somatic mutation in lung, liver and kidneys (Liviac et al., 2010, Oliveira et al., 2007). Human exposure to environmental agents is reported to induced carcinogenesis in many tissues e.g., lungs, skin, breast, ovaries, brain and blood (Abel and DiGiovanni, 2015).

Since genetic damage is implicated in the etiopathogenesis of environmental induced cancer, hence measuring the extent of genetic damage is applied in cancer screening

assays. In cancer risk assessment assays e.g., ames test, comet assay and micronucleus assay, the extent of adduct formation, DNA cross-linking and mutations are investigated. Comet (single cell gel electrophoresis) assay is conducted on cells in order to assess whether a chemical has induced genetic damage or not, and serves to detect DNA breaks. Micronucleus arises from whole lagging chromosomes (aneugenic event) or acentric chromosomal fragment detaching from the chromosome after breakage (clastogenic event). These aneugenic and clastogenic events are assessed by the micronucleus assay. Both the comet and micronucleus assays are used to investigate the intensity and nature of DNA damage which might lead to carcinogenesis (Hussain et al., 2007, Rundle, 2006, Poirier, 2004).

1.2.2. Non-genetic mechanisms in carcinogenesis

Environmental agents-induced DNA damage is an important early event during the initiation phase of carcinogenesis, which reflects a permanent and irreversible change in the initiated cells (Pitot, 2007, Loeb and Harris, 2008). However, recent studies have reported that initiation *per se* in classical carcinogenesis model is not sufficient for tumor development, which results from broader alterations in the cellular homeostasis, mainly because of the inability of initiated cells to properly control and regulate the gene expression (Shiao, 2009). Exposure to environmental stressors, in addition to their genetic effects, also involve a variety of non-genotoxic (i.e., epigenetic effects) effects in cells (Tryndyak et al., 2006). These epigenetic effects also play key role in cancer development. Evidence suggests that epigenetic alterations in cells could result in the emergence of reprogrammed cells with characteristics similar to the cancer cells (Bombail et al., 2004) .

Thus epigenetics is implicated to play critical role in different phases of multistage model of carcinogenesis. For example, traditionally the genetic components such mutations inβ-catenin and P53 were implicated in the carcinogenesis of hepatocellular carcinoma (HCC). With recent surge in epigenetic knowledge, however, it is now accepted that epigenetic factors play critical role in all stages of HCC carcinogenesis (Pogribny and Rusyn, 2014). Further, disruptions in epigenetic pathways are implicated in the carcinogenesis of prostate cancer, retinoblastoma, non-small cell

lung cancer, breast cancer, leukemias, renal carcinomas, colon and endometrial carcinomas. Cellular epigenetic pathways are susceptible to dysregulation by exposure to environmental agents, thus leading to the epigenetic carcinogenesis (Sugimura and Ushijima, 2000, Herceg and Vaissiere, 2011). In order to understand how environmental factors disrupt the epigenetic pathways that lead to epigenetic carcinogenesis, it is thus important to know the components of cellular epigenetic machinery.

1.3.Epigenetics

The term "epigenetics" was coined by Conard Waddington (Waddington, 1942). In simplest term, epigenetics is defined as cellular events, which aren't of genetic origin. This means epigenetic events are not defined by the DNA sequences. Epigenetics is described as the cellular events that act as an interface between genes and environment that influence the cell functions without changing the DNA sequence. As an example, cellular differentiation during development could be considered as an epigenetic phenomenon. During development, cellular differentiation leads to the emergence of multicellular organisms with cells genetically similar but structurally and functionally heterogeneous. This cellular heterogeneity is influenced by the epigenetic mechanisms, which regulate and maintain different patterns of gene expression in cells. These differential gene expression patterns are transmitted during mitosis to successive generations. Thus epigenetics can be defined as stable and heritable changes in gene expression occurring without changes in the DNA sequence (Goldberg et al., 2007).

The covalent and non-covalent modifications of DNA and histone proteins and non-coding-RNAs are the main mechanisms which are involved in the cellular epigenetic machinery (figure 2) (Kanwal and Gupta, 2012). Disruptions in these mechanisms are shown to be linked with epigenetic carcinogenesis. Histone modifications and non-coding-RNAs and their involvement in carcinogenesis is described below briefly, followed by more detailed description of DNA methylation and it is implications in carcinogenesis.

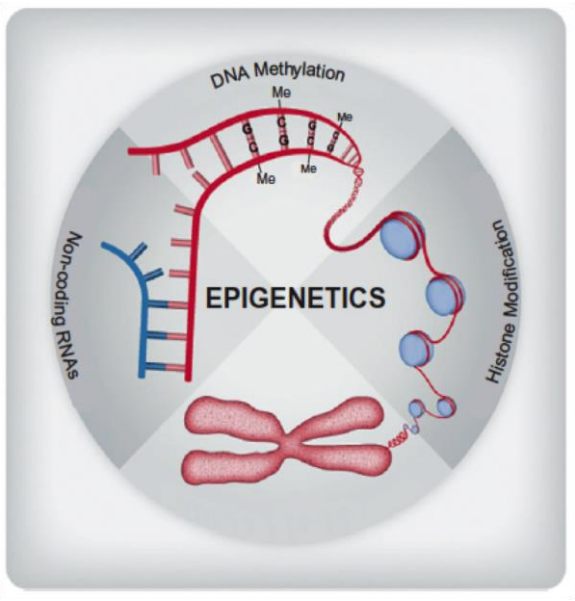

Figure 2: Major categories of epigenetic mechanisms. Cells epigenetic control is mediated by difference mechanisms operating in the cells e.g., DNA methylation, histone modification and noncoding RNAs. These mechanisms confer additional control of gene regulation. Adopted from Sweatt et al., 2012 (Sweatt et al., 2012).

1.3.1. Histone modifications

In eukaryotes, chromatin is a highly condensed structure sequestered in nucleus. It exists in two functionally distinct forms: a condensed form called heterochromatin which occurs during mitosis and meiosis, and decondensed or loose form called euchromatin. Structurally, chromatin is packed in nucleus by repeating units of nucleosomes. Nucleosomes consist of DNA which is wrapped around core histones. Within a nucleosome there are two units of each of the core histones H2A, H2B, H3, and H4. Nucleosomes are considered as the warehouse of epigenetically inherited information. Epigenetic information in this warehouse is stored in the form of covalent modifications of the core histones of nucleosome. Amino terminal of core histone proteins has flexible and highly basic tail region, which is subject to various post-transcriptional modification (PTMs). There are at least 60 different amino acids residues where PTMs has been detected, and these numbers are likely to grow. There are at least eight different types of histone modification: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization. These PTMs regulate the epigenetic information via altering the nucleosome-nucleosome interactions and/or the nucleosome-DNA interactions.

Histone PTMs act in interdependent manner and forms so called the histone code which are the units of cells epigenetic programming (Zhang and Reinberg, 2001, Kouzarides, 2007).

During epigenetic mechanism of carcinogenesis, aberrations in the histone codes arise which can lead to cellular transformation. With decades of research, it is now known that there are patterns of histones PTMs alterations that lead to epigenetic carcinogenesis. For example, lymphocytes of cancer patients showed significantly lower levels of global histone H4 trimethylation (H4K20me3) and acetylation (H4K16ac) compared to the lymphocytes from healthy individuals. The progressive loss of histone H4 trimethylation was further confirmed in the animal model of carcinogenesis. Global loss of H4K20me3 represents an early event in tumorigenesis that arise early in carcinogenesis, and this global loss of H4K20me3 becomes more evident during the sequential progression of carcinogenesis moving from cell hyperplasia to metaplasia, dysplasia, and then to carcinoma in situ (Van Den Broeck et al., 2008).

Histone PTMs are also susceptible to aberrations by exposure of environmental agents. In systematic literature review on environmental exposure induced histone PTMs changes, it was shown that expression of H3Me3K4 and H3Me2K9 histone marks were most abundantly affected by exposure of heavy metals and ethanol (Dik et al., 2012). Cells exposed to these environmental stressors lead to the altered expression level in a number of genes via altering the histone PTMs (Martinez-Zamudio and Ha, 2011). Since alterations in histone PTMs patterns are implicated in carcinogenesis, it is thus foreseen that exposure of environmental stressors can lead to carcinogenesis by altering the histone PTMs.

1.3.2. microRNA

The process of transcription in cells produces large numbers of RNA molecules. Not all of these RNAs are translated into proteins, but rather play regulatory functions in cells, and are called non-coding RNAs (ncRNA). (Kaikkonen et al., 2011). MicroRNAs (miRNA) are the most well-known of the regulatory non-coding RNA

classes. They are transcribed in the nucleus by polymerase II as long primary transcripts (pri-miRNA) up to 1000 nucleotides (nt) in length. pri-miRNA is then cleaved producing a pre-miRNA molecule of approximately 70-100 nt in length. pre-miRNA is transported to the cytoplasm where it undergoes final processing step which produces a duplex molecule containing the single-stranded mature miRNA molecule and a miRNA* fragment. The miRNA:miRNA* complex is incorporated into the RNA-induced silencing complex (RISC). miRNAs regulate gene expression by silencing the target mRNA, and thus repressing expression of target genes (Hayes et al., 2014).

MicroRNAs are involved in the regulation of gene expression at multiple levels. Loss of microRNAs-controlled gene expression is implicated in carcinogenesis. A large number of microRNAs e.g., mir-21, miR-22, miR-200 family, miR-128, miR-193, miR-34a, miR-451, miR-341, miR-320, are involved in different types of cancer (Takahashi et al., 2014, Jansson and Lund, 2012). MicroRNAs act like tumour suppressor genes and proto-oncogenes, and loss of their activity is implicated in carcinogenesis.

With their modulating effects on expression of target genes, microRNA perturbations in response to environmental stressors can lead to altered gene expression signature which could predispose cells to disease conditions. Environmental exposure has shown to affect the expression levels of miRNAs. For example, in a study of Wang., F et al 2014., they reported alterations in 69 microRNAs in response to volatile organic compounds exposure in vivo (Wang et al., 2014). Recently, there has been a great interest in profiling blood-based circulating miRNAs that are proposed to act as carcinogenic markers (Madhavan et al., 2013). miRNAs (e.g., miR-27a, miR-27b, miR-122, miR-148, miR-155, miR-192, miR-214, miR-221, miR-429, and miR-50) are suggested as exposure markers of aflatoxin induced hepatocellular carcinoma (HCC) (Valencia-Quintana et al., 2014). In one study Baccarelli, A et al 2012 (Baccarelli et al., 2012) showed that exposure to particulate matter alters the expression of miR-100 which may regulate genes involved in particulate matter-induced physiological changes. These studies have shown that miRNAs expression is affected by exposure to environmental stressors, which could drive cellular carcinogenesis.

1.3.3. DNA methylation

DNA methylation is a key element among various epigenetic mechanisms. DNA methylation occurs when a methyl (CH_3) group is attached covalently at the carbon 5 (5C) position of the cytosine ring of CpG dinucleotides. The cytosines (C) targeted for methylation are almost exclusively followed by a guanine (G), which are called CpG dinucleotides (Clark et al., 1995). Statistically CpG dinucleotides are underrepresented, and are unequally distributed in the human genome (Varriale and Bernardi, 2010). These CpGs are heavily methylated in the genome. Certain regions, however, in human genome have high density of CpG dinucleotides. These CpG rich regions are called CpG islands (CGIs). CGIs are, on average, 1000 base pairs (bp) long, and show an elevated G+C base composition. High proportions of CGIs are located in the gene promoter regions and first exon of the genes where they are largely unmethylated. Certain CpG islands are occasionally located within the body of the gene, or even in the 3'-region where, they are normally more prone to methylation (Esteller, 2002). Considerable amount of CpGs are also found in repetitive DNA elements such as transposons and retrotransposon-like elements. Repetitive elements make up a large fraction (about 40–50%) of genome, and have usually high CpG content, with CpGs being heavily methylated. Highly methylated CpGs in these regions are necessary to silence the unwanted expression of retrotransposable elements, and hence to maintain the genome stability (Ehrlich et al., 1982).

DNA methylation (5mC) at CpG sites regulates the gene expression. Recent work has also shown that CGIs located remote form the transcription start sites (TSS), also show evidence for gene-promoter functions, which emphasize the correlation of CGIs with gene expression. Thus, DNA methylation plays key role in regulation of gene expression. Methylation poor CpGs promote more relaxed and open chromatin structures that favour gene expression. While, methylated CpGs promotes highly condense chromatin structure where gene expression is repressed. Methylated cytosines at CpG dinucleotides recruit methyl-DNA binding proteins. These proteins have both a methyl-DNA binding domain (MBD) and a transcription-regulatory domain (TRD). The TRD recruits other proteins which such as paired amphipathic

helix protein (Sin3a), and these adaptor proteins in turn recruit histone deacetylases (HDACs) to the site. The HDACs alter chromatin structure locally by removing acetyl groups from histone core proteins, leading to compaction of chromatin and transcriptional suppression. This cascade of events leading to gene expression regulation is depicted in Figure 3a. When CpG are unmethylated (figure 3b), polymerase enzymes bind to DNA that leads to gene expression (Rountree et al., 2001, Bienvenu and Chelly, 2006).

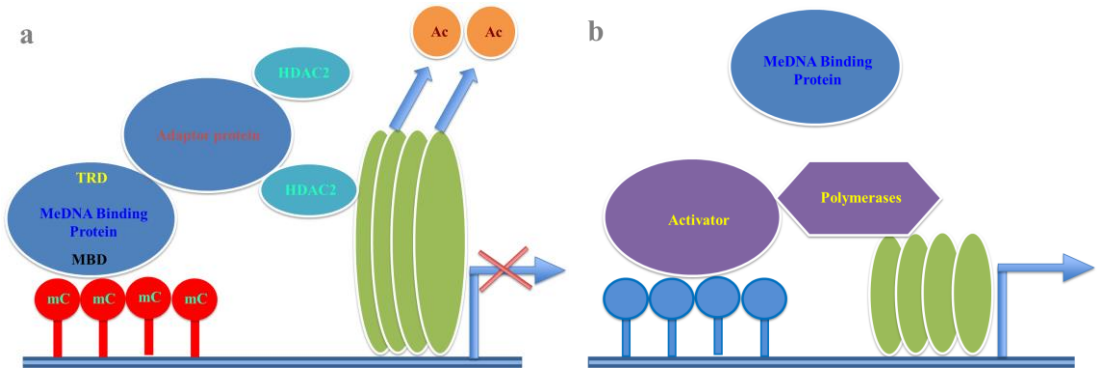

Figure 3: DNA methylation dependent gene silencing. Figure gives a simplified overview of DNA methylation dependent gene silencing. a): Methylated CpGs (red lollipops) recruit methyl-DNA binding proteins which have a methyl-DNA binding domain (MDB) and a transcriptional-regulatory domain (TRD). TRD in turn binds with histone deacetylateses (HDACs) that alter the chromatin structure locally by removing acetyl groups (Ac) from core histones (green spheres). Whole process leads to suppression of gene expression. b): when the CpGs are not methylated (green lollipops), it allows binding of activator proteins which recruits polymerase enzymes that leads to gene expression (Bienvenu and Chelly, 2006).

1.3.3.1. Regulation of DNA methylation

Methylation of DNA is regulated by a class of enzymes known as DNA methyltransferases (DNMTs). Two different types of DNMTs are reported knows as: maintenance DNMTs and de novo DNMTs. DNMT1 is the maintenance DNMT, DNMTs 3a and 3b are the de novo DNMT isoforms. De novo DNMTs methylate previously unmethylated CpG sites in DNA; sites which have no methyl-cytosine on either DNA strand, and thus setting new pattern of DNA methylation. The maintenance DNMT isoform methylates hemimethylated DNA. Thus different DNMTs serve distinct roles in the cell. De novo DNMTs place new methylation marks on DNA, when specific genes are first silenced as part of cell fate determination. Maintenance DNMTs maintains the cellular methylation fidelity after

cell division. They regenerate the methyl-cytosine marks on the newly synthesized complementary DNA strand that arises from DNA replication (Robertson, 2001). Besides DNA methylation, other epigenetic modifications such as 5-hydroxymethylcytosine (5hmC), have been recently discovered. 5hmC is shown to be intermediate of active cellular demethylation mediated by Ten-eleven translocation methylcytosine dioxygenase (Tet) enzymes. Significant levels of 5hmC have been found in DNA from embryonic stem cells, neurons and brain (Shock et al., 2011). The biological functions of 5hmC are not well characterized yet, but it has already showen to be an important pathway in the cellular demethylation machinery (Pastor et al., 2013).

1.3.3.2. DNA methylation in carcinogenesis

Physiological distribution of tissue-specific DNA methylation marks is altered early during the process of carcinogenesis, which is manifested in many pathological conditions. In physiological state, tumour suppresser genes are kept activated by gene hypomethylation; whereas oncogenes and DNA reparative elements are kept silenced by gene hypermethylation in order to maintain genome integrity (Esteller, 2002). In pathological state, tumour suppressor genes are silenced, while repetitive elements and oncogenes are active by aberrant region-specific DNA hypermethylation and genome-wide DNA hypomethylation marks respectively (figure 4).

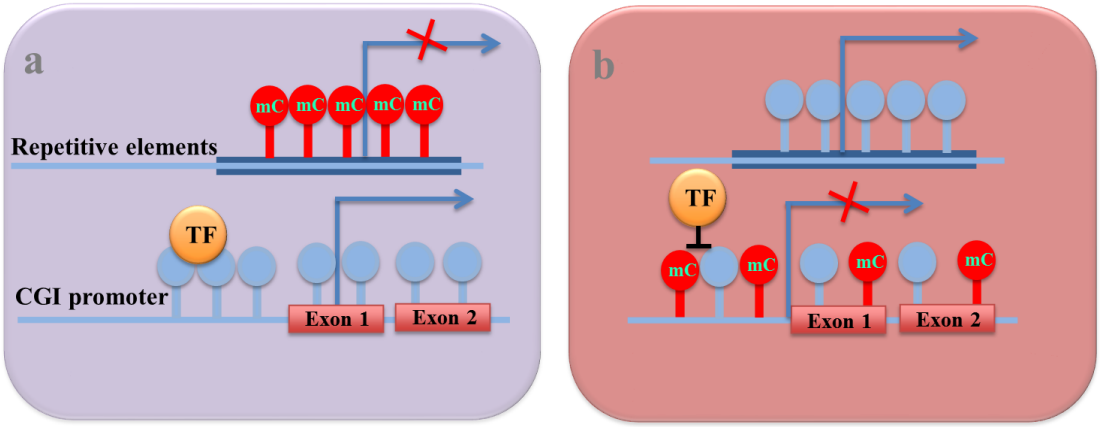

Figure 4: DNA methylation in normal and cancer cells. a) in normal cells repetitive elements are methylated at CpG dinucleotides (red lollipops), while CpG islands (CGI) at promoter sites are not methylated. b) in cancer cells, repetitive elements get aberrantly hypomethylated while and CpGs at

promoter sites are hypermethylated, which hinders the binding of transcription factors (TF) and leads to suppression of tumour suppressor genes activity (Varela-Rey et al., 2013).

The occurrence of DNA methylation aberrations early during carcinogenesis suggests that epigenetic alterations may precede the classical transforming events, such as mutations of tumor-suppressor genes and amplification of oncogenes. This early occurrence of DNA methylation aberrations in carcinogenesis were shown by Kanai et al 1996. It was known that genetic aberrations are involved in hepatocellular carcinomas. But the involvement of DNA methylation aberrations in the pre-cancerous liver tissue i.e., chronic hepatitis or liver cirrhosis, were largely unknown. Later on, aberrant DNA methylation marks were reported in the pre-cancerous (i.e., chronic hepatitis or liver cirrhosis) liver tissue at multiple loci, compared with normal liver tissue, which drives the pre-cancerous liver tissue to carcinogenesis. This indicated the occurrence of DNA methylation aberrations early during the multistage hepatic carcinogenesis (Kanai et al., 1996). In fact, aberrant DNA hypermethylation has been reported in the promoter regions that silence tumour suppressor genes such as *CDKN2A* (cyclin-dependent kinase inhibitor 2A), *CDKN2B* (cyclin-dependent kinase inhibitor 2B), *TP73* (tumor protein p73), *MLH1* (mutL homolog 1), *APC* (adenomatosis polyposis coli), *BRCA1* (breast cancer 1), *MGMT* (O-6-methylguanine-DNA methyltransferase), *VHL* (von Hippel-Lindau tumor-suppressor), *GSTP1* (glutathione S-transferase pi 1), *CDH1* (cadherin 1) and *DAPK1* (death-associated protein kinase 1) (Sharma et al., 2010). These observations highlighted the importance of DNA methylation patterns in functioning of tumour suppressor genes. Aberrations in their methylation patterns were shown to drive the process of carcinogenesis. Since the DNA methylation is a reversible epigenetic modification, it is expected that aberrant DNA methylation can be reversed in order to restore the physiological levels of gene expression. By characterizing the factors which trigger aberrant DNA methylation, it will be possible to build strategies to circumvent such factors, and to develop therapies to reverse the DNA methylation aberrations.

1.3.3.3. DNA methylation and environmental exposure

Numbers of environmental stressors are shown to induce DNA methylation aberrations. Earlier studies linking effects of environmental stressors to epigenetics were conducted by investigating the effects of metals on DNA methylation. Nickel is known to be a human carcinogen, and its exposure is shown to be associated with nasal and lungs cancer via largely unknown mechanisms. Recent investigations showed the involvement of epigenetic pathways in the nickel-induced carcinogenesis. It is now reported that DNA methylation deregulations leading to activation and/or suppression of gene expression, are the early events in the nickel-induced carcinogenesis. Further animal studies showed that the exposure to nickel leads to DNA hypermethylation-induced gene inactivation (Lee et al., 1995). Exposure to other metals e.g., chromium, cadmium, and cobalt, is also shown to induce DNA methylation aberrations (Ziech et al., 2010a). All these findings corroborate the concept that exposure to environmental stressor induce DNA methylation deregulations, which might play an important role in the process of carcinogenesis. The presence of large number of stressors in the environment and their potential impact on cells epigenetic patterns lead to the foundations of relatively new scientific discipline called environmental epigenetics. The effect of environmental stressors i.e., chemicals and nanomaterial, on DNA methylation patterns are discussed further in the section of environmental epigenetics.

So far we have mainly emphasized on epigenetic pathways involved in environmental exposure induced carcinogenesis. However, epigenetics factors are also shown in the aetiology of other disease e.g., cardiovascular, pulmonary and neurobehavioral diseases (Baccarelli and Ghosh, 2012, Schwartz, 2010, Conradt et al., 2013). In the following chapter, we will give a short introduction on the agents which will be studied in this thesis e.g., hydrocarbons, halocarbons, cytostatic agents and nanomaterial. Important findings known, thus far, about the adverse health effects induced by these agents are discussed below. Here, we will focus on the known carcinogenic effects of agents that are included in the current research.

1.4.Environmental epigenetics

Environmental epigenetics encompasses studying the effects/alterations induced by exposure of environmental stressors to the epigenome. Humans are exposed to a wide variety of environmental stressors from different source e.g., exposure to chemicals, organic solvents, metallic dust, smoking, combustion, volcanic eruptions, particulate matter (PM) etc. Human exposure to environmental stressors has been changed dramatically with anthropogenic factors, and more recently with the advents in nanotechnology. In nanotechnology, materials with (nano)size-dependent properties called engineered nanoparticles (NPs) (one dimension <100nm) are manufactured, which represent entities of potential human exposure (Oberdorster et al., 2005). The effect of various environmental stressors on epigenetic mechanisms i.e., histone PTMs and microRNAs is discussed briefly in pervious sections. Here, the effects of various environmental stressors on one of the most studied epigenetic modifications i.e., DNA methylation, are discussed.

1.4.1. Exposure to environmental stressors

It is well known that disease susceptibility is influenced by complex interplay between genetics and environmental factors (Tang and Ho, 2007). In vitro, animal and human studies have identified several environmental agents that may mediate adverse effects through epigenetic mechanisms. Recently, there has been a great interest on studying how the environmental agents lead to epigenetics alterations. Environmental stressors represent a broad group of agents to which humans are exposed. Chemicals and nanomaterial present in the environment are such group of stressors that have the potential to alter the epigenetic patterns. Here, we will discuss the current knowledge linking the exposure of chemicals and nanomaterial with the adverse health effects.

1.4.1.1. Exposure to hydrocarbons, halocarbons, cytostatic agents

Exposure to environnemental stressors i.e., PAHs, halocarbons, cytostatic agents etc., occurs via inhalation, skin contact, ingestion etc. PAHs are ubiquitously present in the

environment. Exposure to PAHs has been linked to a variety of adverse health effects, including cancer. Numbers of PAHs are known mutagens, carcinogens and/or developmental toxicants. For example, benzo[a]pyrene, a representative of PAHs, exerts all three types of adverse health effects. On mechanistic levels, PAHs are shown to induce DNA damage, which accounts for their carcinogenicity (Perera et al., 2005). Through decades of research, a wide variety of chemicals e.g., halogenated hydrocarbons (carbon tetrachloride, trichloroethylene etc), cytostatic agents (e.g., mitomycin C), acrylamide etc, are characterized for the genotoxic potential. Recently, studies have reported that the carcinogenic effects induced by many chemicals e.g., 2-acetylaminofluorene, tamoxifen, trichloroethylene, aflatoxin B1, ochratoxin, nickel and chromium, do not follow a classical genotoxic carcinogenesis model, but it was suggested that they might involve a spectrum of cellular alterations encompassing epigenetics. (Salnikow and Zhitkovich, 2007, Bagny ukova et al., 2008, Marin-Kuan et al., 2008, Shiao, 2009). Current knowledge, however, is insufficient in linking exposure-induced epigenetic changes that might leads to carcinogenesis in premalignant tissue (Carbone et al., 2004).

1.4.1.2. Exposure to organic solvents

Organic solvents represent a class of environmental stressors which confer risk for human diseases (Ziech et al., 2010b). Volatile and the lipophilic nature of organic solvents make them toxicologically important. Being volatile, organic solvents rapidly contaminate the working environment and pose a major health risk. Exposure to organic solvents is shown to be carcinogenic via induction of DNA damage through the production reactive oxygen species (ROS) (Zhang et al., 2009). Exposure to organic solvents is shown to induce DNA damage in cells. Organic solvent exposure induced DNA damage may leads cells to carcinogenesis. Some preliminary studies have also implicated changes in DNA methylation in organic solvents induced carcinogenesis (Liu et al., 2011). These findings lead us to investigate if human exposure to organic solvents involves DNA methylation changes, which might lead to carcinogenesis.

Owing to the lipophilic nature of organic solvents, body tissues especially the neurons are prone to the detrimental effects of solvents exposure. A specific syndrome, called chronic toxic encephalopathy (CTE) has been known for many years, clinically expressed as e.g., nausea, dizziness, fatigue, headache, short-term memory loss, attention impairment and personality changes (Arliensoborg et al., 1979, van Valen et al., 2009). Genetic polymorphisms in biotransformation enzymes play important role in modifying response towards organic solvents exposure. *CYP2E1*5B, GSTPE*1C* and *EPHXI* exon 4 genotypes are important in modifying the CTE risk in organic solvent exposed individuals (Kezic et al., 2006), yet do not fully describe the phenotype of acquired neurobehavioral disorders such as CTE. Recent evidence suggested epigenetic mechanisms might provide answers in the aetiology of CTE in individuals exposed to organic solvents.

1.4.1.3. Exposure to nanomaterial

With recent developments in the field of nanotechnology, a new class of environmental stressors called engineered nanoparticles has emerged (Medina et al., 2007). NPs are defined as particles with at least one dimension less than 100 nm (Oberdorster et al., 2005). Humans exposure to nanomaterial occurs by different route e.g., inhalation, skin contact, ingestion. Also, there are different types of nanomaterial to which humans are exposed (Hoet et al., 2004).

Nanomaterial e.g. gold NPs (AuNPs), titanium dioxide NPs and single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) have gained widespread applications. Owing to their widespread applications, humans exposure to nanomaterial has also changed (Yokel and Macphail, 2011). Once inside the body, the clearance and long-term fate of AuNPs and CNTs is not well characterized. Preliminary studies have reported tissue specific accumulation of nanomaterial in body (Choi et al., 2010, Tang et al., 2009). Apart from their small size, nanomaterial have other facets with associated toxicological profiles. For example, AuNPs and CNTs can be functionalized with different polymers to add specific physicochemical properties to them. This feature can also add up towards their toxicological profile (Love et al., 2012).

Number of studies has reported the cellular, immune and genotoxic deregulations associated with AuNPs and CNTs exposure in in vitro and in animals models (Love et al., 2012). In some of these reports, contradictory results regarding to the toxicological endpoints associated with AuNPs and CNTs exposure are reported (Yildirimer et al., 2011). This is understandable, since AuNPs and CNTs used across these studies differ in their physicochemical characteristics. Of particular interest are the CNTs, which are assumed to present physicochemical properties similar to asbestos (Pacurari et al., 2010). One study reported the carcinogenic potential of CNTs, but results of that study were not replicated in further studies (Pacurari et al., 2010, Lohcharoenkal et al., 2014). Studies conducted on assessing toxicological properties of nanomaterial are mainly focused on classical cellular endpoints i.e., cytotoxicity, immunetoxicity and genotoxicity assays (Yildirimer et al., 2011). Nanomaterial effects on cells epigenetic status have not been investigated. Since cells epigenetic machinery is susceptible to alterations in response to exposure of environmental agents (Szyf, 2011), it is expected that cells exposure to AuNPs and CNTs could also lead to the altered patterns of DNA methylation. AuNPs and CNTs induced DNA methylations have not been investigated previously. It is, hence, proposed that DNA methylation could be an important epigenetic factor that regulates the gene expression on cellular exposure to AuNPs and CNTs. Since the larger counterpart of nanomaterial, i.e., particulate matter (PM) is shown to induce DNA methylation deregulations (Hou et al., 2011), nanomaterial exposure could also lead to similar epigenetic changes in cells.

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2. Chapter 2

2.1.Study objectives

Humans are chronically exposed to different chemicals in the environment, such as organic solvents, hydrocarbons and halocarbons and cytostatic agents. With recent advancements in the field of nanotechnology, there has emerged new category of environmental stressors called nanomaterial. Exposure to these environmental stressors is shown to induce adverse cellular effects under different experimental settings. For example, exposure to number of chemicals and nanomaterial has shown to induced cytotoxic and genotoxic effects. Recently, there has been great interest in understanding the epigenetic effects associated with exposure to these environmental agents. Cells epigenetic changes after exposure to environmental stressor does not necessarily means that cells are undergoing to disease process. But rather, epigenetic changes induced by environmental agents could be a part of cells normal physiological response. Hence, it is important to understand cells epigenetic response to environmental agents, and to differentiate the epigenetic changes associated with cells normal physiological response, from epigenetic changes leading to pathological response. Thus characterizing epigenetic changes in response to exposure of environmental agents are important in understanding aetiology of environmentally induced diseases. Current project is set-up to fill this knowledge gap; that to characterize the epigenetic changes associated with exposure to the environmental stressors. The aims of the project are summarized below.

2.1.1. To investigate the epigenetic effects of environmental stressors

Epigenetic effects of chemicals exposure were investigated in two phase i.e., a laboratory phase and a human phase. The epigenetic effects of exposure to nanomaterial were investigated in rodent model.

2.1.1.1. Exposure to organic solvents, hydrocarbons, halocarbons, cytostatic agents in vitro

In the laboratory phase, epigenetic alterations were investigated in vitro induced by exposure to different classes of chemicals e.g., organic solvents, polyaromatic hydrocarbons, halocarbons, and cytostatic agents. It was hypothesized that exposure of these carcinogens in vitro would induce global DNA methylation alterations similar to ones observed in cancer cells. Study was conducted in TK6 cells exposed to different chemicals at three dose levels for 24 hours. Preliminary cell viability assays were performed in order to select the exposure dose, and global DNA methylation was quantified by LC-MS/MS method.

2.1.1.2. Exposure to organic solvents in humans

In vitro findings, of DNA methylation changes in response to organic solvents exposure, were further translated in human population exposed to solvents. In the human phase, hypothesis was investigated whether solvent exposure in humans would alter their blood cells DNA methylation patterns. Also, gene-environment interactions were investigated by analysing the association between global DNA methylation and genotypic difference in drug metabolizing enzymes.

2.1.2. Exposure to nanomaterial

Epigenetic effects induced by exposure of nanomaterial were investigated in mice exposed to gold nanoparticles and carbon nanotubes.

2.1.2.1. Exposure to gold nanoparticles and carbon nanotubes

Mice were exposed to gold nanoparticles of three primary sizes of 5 nm, 60 nm, and 250 nm, and were also exposed to two types of carbon nanotubes; single walled carbon nanotubes and multi walled carbon nanotubes. Hypothesis was tested whether exposure of gold nanoparticles and carbon nanotubes to rodents would induce global DNA methylation and hydroxymethylation changes similar to ones observed in cancer cells. Gene promoter methylation changes were also investigated in rodents in response to nanoparticles and carbon nanotubes exposure, and it was hypothesized

that gold nanoparticles and carbon nanotubes exposure in rodents would lead to alterations in their gene promoter methylation as well. Cellular pathways often responsive to environmental exposure e.g., oxidative stress pathway, immune pathway, cell cycle regulation pathways, DNA methylation pathways; were selected based on the literature search. Within these pathways, we selected nineteen candidate genes with reported gene promoter alterations in response to xenobiotic exposure. PCR-pyrosequencing based gene promoter methylation assays were developed for the selected genes to investigate changes in their promoter methylation in mice lung and blood DNA samples exposed to nanomaterial. List of selected genes with their respective PCR-pyrosequencing assays is given in chapter 5.

The aims and objectives of current research were investigated and results are presented in three manuscripts. Each manuscript is represented by a chapter (chapter 3, 4 and 5) in this thesis. In order to overcome the technical issues in testing hypothesis, techniques and methods were developed where required. Methods development and validations are published in two research articles, which are represented in this thesis as two chapters (chapter 6 and 7).

In chapter 3, global DNA methylation patterns associated with exposure to a wide range of chemical stressors were investigated in lymphoblastic (TK6) cells. TK6 cells have wild type *P*53, grow in suspension and have short doubling time. These characteristics make TK6 cells suitable for toxicological screening of environmental stressors (Brehwens et al., 2010). In chapter 4, epigenetic effects induced by exposure to organic solvents in human blood were investigated. Blood comprises epigenetically different subpopulations of cells, and since epigenetic patterns are tissue specific, use of blood as surrogate tissue in epigenetic toxicology is greatly discussed (Horvath et al., 2012, Terry et al., 2011). In chapter 5, epigenetics effects induced by exposure to nanomaterials in BALB/c mice were investigated. BALB/c mice are inbred strain which offers homogenous response to xenobiotic exposure. Balb/c mice are commonly used in studies investigating the effects of environmental stressors (Rosenfeld, 2010).

Several techniques are made available which can be used to analyse the epigenetic variations associated with environmental exposure. Use of techniques, to some extent,

is based on the expected magnitude in epigenetic variations under investigations. For example, if the magnitude of variations is expected to be low, the choice of analytical methods is limited to those with high sensitivity to quantify these variations. Also, some techniques allow the quantification of DNA methylation at global level and other quantify at single base resolution (Zuo et al., 2009). In current research, quantification of global DNA methylation and hydroxymethylation was performed with liquid chromatography – tandem mass spectrometry (LC-MS/MS). LC-MS/MS methods for global DNA methylation and hydroxylmethylation quantification was developed and optimized. Development and validation of method is described in chapter 6. For gene promoter methylation, highly quantitative bisulfite-PCR pyrosequencing assays were developed and optimized for rodent genome. Details of bisulfite-PCR pyrosequencing method are described in chapter 7.

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3. Chapter 3

Adopted from:

Epigenetic factors in cancer risk: effect of chemical carcinogens on global DNA methylation pattern in human TK6 cells

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Abstract

In the current study, we assessed the global DNA methylation changes in human lymphoblastoid (TK6) cells *in vitro* in response to 5 direct and 10 indirect-acting genotoxic agents. TK6 cells were exposed to the selected agents for 24 h in the presence and/or absence of S9 metabolic mix. Liquid chromatography-mass spectrometry was used for quantitative profiling of 5-methyl-2'-deoxycytidine. The effect of exposure on 5-methyl-2'-deoxycytidine between control and exposed cultures was assessed by applying the marginal model with correlated residuals on % global DNA methylation data. We reported the induction of global DNA hypomethylation in TK6 cells in response to S9 metabolic mix, under the current experimental settings. Benzene, hydroquinone, styrene, carbon tetrachloride and trichloroethylene induced global DNA hypomethylation in TK6 cells. Furthermore, we showed that dose did not have an effect on global DNA methylation in TK6 cells. In conclusion we report changes in global DNA methylation as an early event in response to agents traditionally considered as genotoxic.

3.1. Introduction

Environmental carcinogens are a known risk factor of human cancer (Ziech et al., 2010). In its classical model, carcinogenesis initiates and proceeds through changes in the genome (i.e., genetic effects) (Vogelstein and Kinzler, 2004). Thus, measuring carcinogen-induced DNA damage i.e., DNA adducts formation and cross-linking, and DNA mutations have been employed in classic cancer risk assessment approaches, e.g., Ames test, comet assay and micronucleus assay (Hussain et al., 2007, Rundle, 2006, Poirier, 2004). Carcinogen-induced DNA damage is an important early event during the initiation phase of carcinogenesis, which reflects a permanent and irreversible change in the initiated cells (Pitot, 2007, Loeb and Harris, 2008). However, initiation *per se* in a classical carcinogenesis model is not sufficient for tumor development, which results from broader alterations in the cellular homeostasis, mainly because of the inability of initiated cells to properly control and regulate the gene expression (Shiao, 2009).

Exposure to genotoxic carcinogens, in addition to their genetic effects, might involve a variety of non-genotoxic effects in cells (Tryndyak et al., 2006). Non-genotoxic effects in cells may play an important role in cancer development (Nakayama et al., 2006). Evidence suggest that non-genotoxic alterations in cells, e.g., alterations in cellular epigenome, could result in the emergence of epigenetically reprogrammed cells (Bombail et al., 2004). These epigenetically reprogrammed cells show an epigenetic profile similar to that frequently observed in cancer cells, such as altered histone modification patterns, hypomethylation of DNA repetitive elements and proto-oncogenes and hypermethylation of tumor suppressor genes. Altered epigenetic status confers genome instability and loss of controlled growth signals, typically observed in cancer cells (Karpinets and Foy, 2005). Epigenetic alterations rather than specific genetic mutations *per se* are reported for the clonal expansion of altered hepatic preneoplastic foci and tumor development (Pogribny et al., 2010).

Recently, a number of studies reported that the carcinogenic effects induced by 2-acetylaminofluorene, tamoxifen, trichloroethylene, aflatoxin B1, ochratoxin, nickel and chromium do not follow a classical carcinogenesis model, but rather involve a

spectrum of cellular alterations encompassing the epigenetics. (Salnikow and Zhitkovich, 2007, Bagnyukova et al., 2008, Marin-Kuan et al., 2008, Shiao, 2009). Epigenetic factors play an important role in cancer etiology; however, there is insufficient knowledge in linking epigenetic factors to environmental carcinogenesis in premalignant tissue (Carbone et al., 2004). Based on increasingly documented epigenetic changes in cancer etiology, the goal of this study is to assess if alterations in global DNA methylation are an early cellular event in response to genotoxic carcinogens with a well-known mode of action (adducts forming and cross-linking agents). In this study, we used 5 direct and 10 indirect- acting genotoxic carcinogens to expose human lymphoblastoid cells (TK6) for 24 h. TK6 cells were exposed to carcinogens at 3 dose levels (low, medium and high) in duplicates. S9 metabolic mix was added in cultures in half of the experiments because indirect- acting carcinogens require S9 metabolic mix to become functional carcinogens. We used human thymidine kinase heterozygote TK6 cells in this study because they express wild-type p53, grow rapidly in suspension (population doubling time of 12–14 h), and are routinely used in genetic toxicology studies. After exposure, cells were harvested, DNA was extracted, hydrolyzed, and global DNA methylation levels were quantified in TK6 cells.

3.2.Materials and methods

3.2.1. Cell culture

TK6 cells were purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). Cells were divided into 15 treatment groups and 2 control groups (control S9-, control S9+), and cultured in RPMI 1640 medium containing 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM l-glutamine at 5% CO2 and 37° C. Cells were maintained at a density of 10^6 cells/ml and exposed for 24 h to carcinogens. We set up two biological replicates per chemical dose, 10 control S9- replicates, and 5 control S9+ replicates.

Due to the requirement of enzymatic biotransformation of procarcinogens to become active carcinogens, a mixture of S9 (1% v/v) from human liver was added to the culture in half of the experiments (van Leeuwen et al., 2005, González Borroto et al., 2001). Liver S9 fractions were obtained from Celsis (Neuss, Germany), and contained drug-

metabolizing enzymes including the cytochromes P450, flavin monooxygenases, and UDP glucuronyl transferases. An exogenous NADPH-regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride; BD Biosciences, Erembodegem, Belgium) required by liver S9 for phase I oxidation was included in the experiments. Cells were exposed to carcinogen in duplicates with or without S9 metabolic mix.

3.2.2. Chemicals, viability assays and dose selection

We selected chemicals with well-described genotoxic characteristics (Tsuda et al., 2000). A list of the selected agents, their classification and exposure dose is given in Table S1. All chemicals were purchased from Sigma Aldrich, and dissolved and diluted in dimethylsulfoxide (DMSO). Viability assays were used to select doses per agent. We used 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) viability assay (Mosmann, 1983), and also counted the proportions of living and dead cells using a Countess^{TM} Automated Cell Counter (Invitrogen, Carlsbad, CA). Based on the viability assays, we selected three doses per chemical, i.e. a dose with 95% cellular viability (high dose), 1/10 of high dose (medium dose) and 1/100 of high dose (low dose).

3.2.3. DNA extraction, concentration and purity

After 24 h of treatment, cells were immediately processed for DNA extraction. DNA was extracted using Trizol® reagent with the PureLinkTM Micro-to-Midi System® according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). DNA quantity and quality was measured by NanoDrop Spectrophotometry and Agilent 2100 bioanalyzer.

3.2.4. Enzymatic hydrolysis of DNA

Extracted DNA was hydrolyzed to individual deoxyribonucleosides in a simplified one-step procedure (Quinlivan and Gregory, 2008). In short, DNA digest mix was prepared

by adding 250 U Benzonase (Sigma Aldrich), 300 mU Phosphodiesterase I (Sigma Aldrich), and 200 U alkaline phosphatase (Sigma Aldrich) to 5 ml Tris-HCl buffer (pH 7.9, 20mM) containing 100mM NaCl and 20 mM $MgCl_2$. 1 µg of extracted DNA from exposed and control samples was hydrolyzed in 100 µl of reaction by adding 50 µl of digest mix, and samples were incubated at 37° C for 6 h. Hydrolyzed samples were brought to 1 ml by adding HPLC-grade H_2O.

3.2.5. Calibration standards

Calibration standards for 5'methyl- deoxycytidine ((5Me)dC) and deoxycytidine (dC) were purchased from Sigma, and dissolved in LC-MS grade water (stock solutions). A calibration series was prepared for 5(Me)dc and dC in a range of 0.1-10 ppb and 10-100 ppb respectively from the stock solutions. The same calibration standards were used in all of the experiments.

3.2.6. LC-ESI-MS/MS instrumental analysis

Global DNA methylation was obtained by quantifying (5Me)dC and dC using ultra-pressure liquid chromatography (UPLC) for fraction separation and tandem mass spectrometry (MS-MS) for quantification. Analyses were carried out on Waters Acquity UPLC equipped with autosampler and Micromass MS Technologies Quattro Premier mass spectrometer. A 10 µl sample was introduced on an Acquity UPLC BEH C_{18}, 50 mm x 2.1 mm, 1.7 µm column, held at 40°C. Mobile phase used for chromatographic separation was a mixture of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) using the following gradient: 0 min: 90% A and 10% B, 2 – 2.5 min: 100% B, 3.9 – 4.0 min: 90 % A and 10 % B at a flow rate of 0.35 ml/min. All mobile phase constituents were LC-MS grade and were purchased from Biosolve (Valkenswaard, the Netherlands).

First, we performed full-scan spectrum under electrospray ionization (ESI) conditions. In full scan spectrum, sodium adducts 5(Me)dC/dC [M+Na]+ and 5(Me)dC-dC dimers were also observed, which is a common phenomenon in an ESI-MS full scan (Song et al., 2004). Analyses were performed in ESI+ mode and a multiple reaction monitoring

(MRM) method was used with argon as the collision gas at a pressure of 2.88 10^{-3} mbar. Transitions monitored were m/z 242.00 → 125.85 for 5(Me)dC (cone voltage 14 V, collision energy 10 eV) and m/z 228.10 → 112.00 for dC (cone voltage 14 V, collision energy 17 eV). Dwell time per transition was 100 ms.

3.2.7. Calibration curve

We observed linear response of standards over a range of concentrations (0.1-10 ppb and 10-100 ppb) for 5(Me)dC and dC with correlation coefficients of 0.9991 and 0.9970 respectively.

3.3. Statistics

The percentage of global DNA methylation was calculated per chemical dose and is expressed as (5Me)dC / [(5Me)dC+dC] %. We used marginal model to explore factors accounted for in the observed variation in global DNA methylation in TK6 cells, i.e., chemicals, dose and S9. Residuals were plotted to verify the assumptions of normality in the marginal model. The Shapiro-Wilk test for residuals was shown to be non-significant, which implied that approximating a response to a normal distribution was appropriate. The SAS 9.2 statistical package was used to fit the marginal model. Box plots were generated for chemicals with a significant effect on global DNA methylation in TK6 cells using SPSS v.18.

3.4. Results

Global DNA methylation in control and exposed cultures per chemical dose without and with S9 metabolic mix is given in Table 1 and 2 respectively. Our results show induction of global DNA hypomethylation in response to S9 metabolic mix as shown in Figure 1.

Table 1: Global DNA methylation in TK6 cells per chemical dose in the absence of S9 metabolic mix.

Chemicals exposed to TK6 cells *in vitro*	Global DNA Methylation in TK6 Cells (S9−) mean, +/− SD		
	Low Dose	Medium Dose	High Dose
Control S9−	6.38, +/−1.21		
Formaldehyde	4.09, +/−0.23	5.21, +/−0.57	4.61, +/−0.23
Styrene	4.67*	3.91, +/−0.09	4.42*
Styrene oxide	6.41*	6.05, +/−0.64	4.95, +/−0.39
Benzene	4.71, +/−0.06	4.31, +/−0.61	4.51, +/−0.03
Hydroquinone	3.71, +/−0.21	3.51, +/−0.42	5.31, +/−0.57
Mitomycin C	7.23*	4.52, +/−0.12	6.35, +/−0.63
Ethylenedibromide	**	3.41, +/−0.37	3.29, +/−0.43
Epichlorohydrin	3.81, +/−0.72	4.44, +/−0.57	4.42, +/−0.62
Acrylamide	5.12, +/−0.08	3.21, +/−0.1	4.72, +/−0.33
Trichloroethylene	**	5.32, +/−0.13	5.91, +/−0.3
Carbon tetrachloride	4.61, +/−0.55	4.21, +/−0.07	4.31, +/−0.02
Cyclophosphamide	**	4.12, +/−0.43	8.24*
Benzo[a]fluoranthene	4.11, +/−0.3	5.84, +/−1.3	**
Benzo[a]pyrene	7.51, +/−1.47	4.36, +/−0.22	7.43, +/−0.94
Benz[a]anthracene	6.55*	3.74, +/−0.08	6.09*

Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.
SD: Standard deviation,
*standard deviation could not be calculated because sample replicates did not pass the quality control,
**global DNA methylation values are not calculated because samples did not pass the quality control.
doi:10.1371/journal.pone.0034674.t001

Table 2: Global DNA methylation in TK6 cells per chemical dose in the presence of S9 metabolic mix.

Chemicals exposed to TK6 cells *in vitro*	Global DNA Methylation in TK6 Cells (S9+) mean, +/− SD		
	Low Dose	Medium Dose	High Dose
Control S9+	4.46, +/−0.83		
Formaldehyde	4.61, +/−0.44	4.55, +/−0.43	4.23*
Styrene	4.49, +/−0.19	3.13, +/−2.35	1.67*
Styrene oxide	5.11*	5.03, +/−0.72	3.33*
Benzene	**	2.92*	3.99, +/−0.05
Hydroquinone	**	4.36, +/−0.37	1.77*
Mitomycin C	5.16, +/−0.51	5.17, +/−0.31	6.22, +/−0.51
Ethylenedibromide	5.24, +/−1.27	4.53, +/−0.06	4.09, +/−0.29
Epichlorohydrin	3.89, +/−0.51	4.62, +/−0.62	3.85, +/−0.14
Acrylamide	3.71*	4.41, +/−0.19	3.95, +/−0.24
Trichloroethylene	3.61, +/−2.65	1.72*	2.59, +/−0.74
Carbon tetrachloride	**	3.86, +/−0.97	3.72*
Cyclophosphamide	**	2.85, +/−1.81	4.93*
Benzo[a]fluoranthene	4.36, +/−0.04	3.38, +/−0.16	4.28, +/−0.65
Benzo[a]pyrene	6.45*	4.85, +/−0.27	5.28, +/−0.07
Benz[a]anthracene	4.16, +/−0.75	4.95, +/−0.66	4.62*

Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.
SD: Standard deviation,
*standard deviation could not be calculated because sample replicates did not pass the quality control,
**global DNA methylation values are not calculated because samples did not pass the quality control.
doi:10.1371/journal.pone.0034674.t002

Variation in global DNA methylation of control and exposed cultures demonstrated normal distribution (Figure S1). Assuming global DNA methylation to be normally distributed, and considering each chemical exposure to be independent but replication within exposure to be correlated, a marginal model, which captures this dependency,

was applied. Covariance between model residuals, which corresponds to uniform correlation within repeated samples, was estimated to be 0.54. Ignoring the correlation within replicated exposures could result in an in accurate estimate of the significance of global DNA methylation.

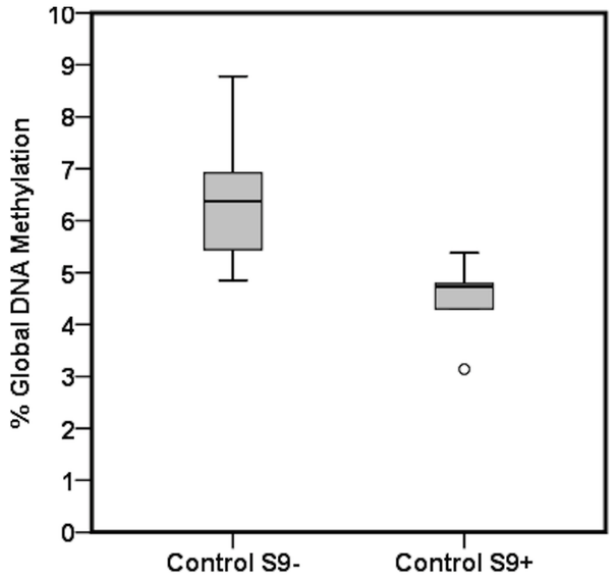

Figure 1: Global DNA methylation in TK6 cells cultured without S9 (control S9−) and with S9 (control S9+) is shown in the box plot.

Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome. The box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as open circles outside the ends of whiskers.

In our results, we observed chemicals and S9 accounting for the observed variability in global DNA methylation in TK6 cells (Table S2). Dose was found to be non-significant even in the absence of S9 in the marginal model. The model was refitted excluding the dose and the results are given in Table 3.

Table 3: The effect of S9 metabolic mix and carcinogens on global DNA methylation in TK6 cells in vitro.

Effect	Estimate	Standard Error	*t*-Value	*p*-Value
S9	−0.9082	0.1956	−4.64	<. 0001*
Formaldehyde	−0.9032-	0.6676	−1.35	0.1806
Styrene	−1.7332	0.6676	−2.60	0.0115*
Styrene oxide	−0.2999	0.6676	−0.45	0.6547
Benzene	−1.5289	0.6877	−2.22	0.0295*
Hydroquinone	−1.8029	0.6877	−2.62	0.0108*
Mitomycin C	0.3268	0.6676	0.49	0.6261
Ethylenedibromide	−0.9566	0.6676	−1.43	0.1565
Epichlorohydrin	−1.2766	0.6676	−1.91	0.0601
Acrylamide	−1.2649	0.6676	−1.89	0.0624
Trichloroethylene	−1.5302	0.6879	−2.22	0.0294*
Carbon tetrachloride	−1.3879	0.6877	−2.02	0.0475*
Cyclophosphamide	−0.4141	0.7163	−0.58	0.5651
Benzo[a]fluoranthene	−0.9712	0.6879	−1.41	0.1626
Benzo[a]pyrene	0.5434	0.6676	0.81	0.4185
Benz[a]anthracene	−0.4332	0.6676	−0.65	0.5186

The table gives parameter estimates and standard errors for a random intercept model with chemicals and S9 as fixed effects.
*Significant at α level of 0.05.
doi:10.1371/journal.pone.0034674.t003

Furthermore, we show that benzene and its metabolite hydroquinone, and styrene, carbon tetrachloride and trichloroethylene significantly affected the global DNA methylation in TK6 cells (Table 3). Global DNA methylation profiles observed with exposure to these chemicals in TK6 cells without and with S9 are shown in Figure 2 and 3 respectively.

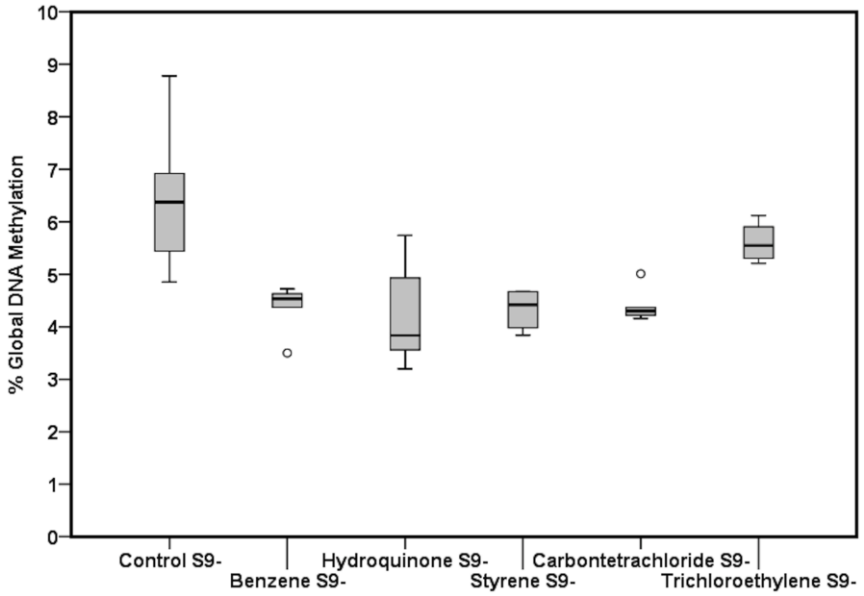

Figure 2: Box plot representation of global DNA methylation in control TK6 cells and TK6 cells exposed with benzene, hydroquinone, styrene, carbon tetrachloride and trichloroethylene without S9 metabolic mix.

Global DNA methylation is expressed as percentage of 5-methylcytosine versus the total number of cytosines present in the genome. The box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as open circles outside the ends of whiskers.

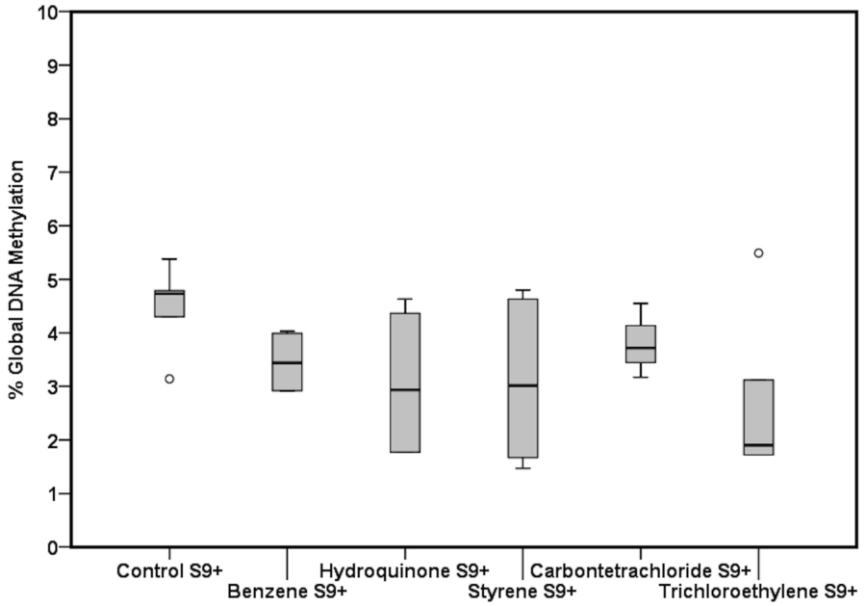

Figure 3: Box plot representation of global DNA methylation in control TK6 cells and TK6 cells exposed with benzene, hydroquinone, styrene, carbon tetrachloride and trichloroethylene with S9 metabolic mix.

Global DNA methylation is expressed as percentage of 5-methylcytosine versus the total number of cytosines present in the genome. The box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as open circles outside the ends of whiskers.

3.5. Discussion

The classical theory of carcinogenesis is driven by genetic mutations and chromosomal abnormalities conferring genome instability (Yamasaki et al., 1992, Parodi et al., 1992). However, the current study highlights the importance of global DNA methylation as an early epigenetic factor in response to genotoxic exposure.

Indirect- acting carcinogens require metabolic activation to become reactive carcinogens. Due to the required metabolic activation, a mixture of S9 liver extract (1% v/v) was added to half of the cultures. S9 mixture contains enzymes required for phase-I metabolic activation of xenobiotics. Expression of metabolic enzymes is linked to reactive oxidative stress pathways (Paolini et al., 2004). Oxidative stress affects DNA methylation by altering the S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) ratio (Panayiotidis et al., 2009, Hitchler and Domann, 2007, Lertratanangkoon et al., 1997). In this study, the addition of S9 metabolic mix in TK6 cell cultures resulted in global DNA hypomethylation (β = -0.9082, p < 0.0001) (Table 3, Figure 1). S9-induced global DNA hypomethylation in these cultures could be mechanistically linked to the induction of oxidative stress pathways. Oxidative stress activates cellular and nuclear signaling pathways, which have intrinsic histone acetyltransferase (HAT) and histone deacetylase (HADC) activities. In turn, these proteins are linked to DNA methyltransferases (DNMTs) in the nuclear pathways leading to the conformational changes in histones and chromatin structure, and thus they alter the cellular transcription level (Ziech et al., 2011, Rahman, 2003).

A number of chemicals used in this study affected global DNA methylation changes in TK6 cells (Table 3, Figure 2 and 3). We observed interesting global DNA methylation patterns. Benzene (β = -1.5289, p < 0.0295) and it metabolite hydroquinone (β = -1.8029, p < 0.0108) exposure induced global DNA hypomethylation in TK6 cells, while styrene exposure (β = -1.7332, p < 0.0115)

induced global DNA hypomethylation but its metabolite styrene oxide exposure did not affect the global DNA methylation in TK6 cells (β = -0.2999, $p < 0.6547$). Benzene exposure has shown to be linked to reduced methylation levels of DNA repetitive elements (Bollati et al., 2007). Benzene and hydroquinone exposure activates the oxidative stress pathways in cells which affects the cellular DNA methylation pattern (Badham et al., 2010). Styrene exposure induces DNA adduct formation and oxidative stress in cells (Harvilchuck et al., 2009). Besides these effects, we report the induction of global DNA hypomethylation by styrene as a potential non-genotoxic mechanism, which could account for its toxicity. We also exposed TK6 cells to carbon tetrachloride and trichloroethylene. These chemicals mainly act through the formation of reactive intermediates after the metabolic activation. In the current study, we observed global DNA hypomethylation induced by carbon tetrachloride (β = -1.3879, $p < 0.0475$) and trichloroethylene (β = -1.5302, $p < 0.0294$) exposure in TK6 cells (Table 3, Figure 2 and 3). Previous studies also reported similar findings about carbon tetrachloride and trichloroethylene (TCE) induced global DNA hypomethylation. Carbon tetrachloride induced global DNA hypomethylation was rescued by supplementation with S-adenosylmethionine (SAM) in rat liver (Varelamoreiras et al., 1995, Tao et al., 1999). These observations suggested that carbon tetrachloride induced DNA hypomethylation involved methionine metabolic pathways. In addition, these chemicals induce oxidative stress, which could affect the cellular methylome.

In contrast to other studies, we did not observe global DNA methylation changes in TK6 cells by exposure to poly-aromatic hydrocarbons (PAHs). Chronic exposure of benzo[a]pyrene to mouse embryonic fibroblasts *in vitro* induced global DNA hypermethylation (Yauk et al., 2008). Also, differences in DNA methylation levels have been reported in peripheral blood lymphocytes of workers chronically exposed to PAH compared to their matched controls (Pavanello et al., 2009). Different experimental settings used in these studies compared to the current study could explain the heterogeneity observed in PAHs induced DNA methylation changes. Furthermore, no global DNA methylation changes in TK6 cells were observed for mitomycin C, formalin, cyclophosphamide, ethylenedibromide, epichlorohydrin and acrylamide. Global DNA methylation changes in response to these chemicals have not been reported elsewhere. Subtle epigenetic effects, such as histone modifications

and gene specific DNA methylation, in response to these chemicals could not be ignored and will be explored further.

Global DNA hypomethylation in TK6 cells induced by direct and indirect- acting genotoxic carcinogens investigated in this study could imply that cells are under pre-neoplastic conditions. If sustained global DNA hypomethylation persists, this could drive these cells to neoplastic phenotype. However, the duration and extent of exposure required for sustained global DNA hypomethylation to confer neoplastic phenotype needs to be fully understood.

Conclusion

In conclusion, we report the non-genotoxic effect, i.e., alteration in global DNA methylation, in response to a number of carcinogens, which are traditionally considered to act through genotoxic mechanisms. We also describe that S9 metabolic mix alters the global DNA methylation pattern in TK6 cells. Future work will address the dose-dependent effects of S9 metabolic mix *in vitro* and the pathways involved in carcinogen-induced DNA methylation changes. Our results suggest the use of different cell lines and more varied assays to validate the above findings, and to explore the mechanistic links.

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Supporting information

Figure S1: Histogram and density plot of residuals to assess normality**.** Normality assumption of response (global DNA methylation) was assessed by plotting the residuals (x-axis). The plot appears to indicate that this assumption is plausible. Shapiro-Wilk test was also performed to confirm normality and residuals were shown to be non-significant.

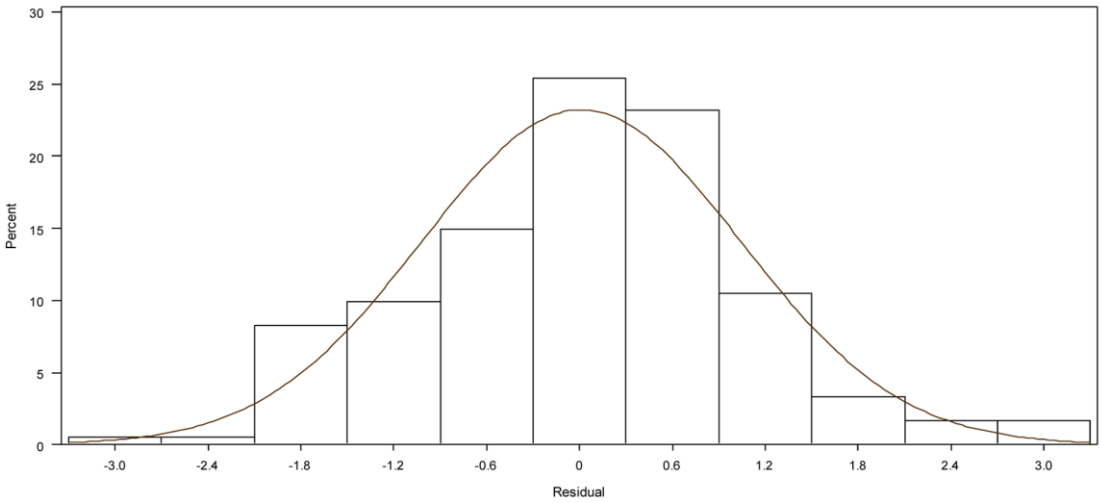

Table S1: Overview of agents, their classification and administered doses used in the treatment of TK6 cells.

Agents	IARC[§]	Category	Concentration (µM)		
			High	Medium	Low
Formaldehyde[*,1,2]	1	Aldehyde	100	10	1
Styrene[**,1]	2B	[a]AH	5000	500	50
Styrene 7,8-oxide[*,1]	2A	[a]AH	500	50	5
Benzene[**,1]	1	[a]AH	100	10	1
Hydroquinone[*,1]	3	[a]AH	0.5	0.05	0.005
Mitomycin C[*,2]	2B	Cytostaticum	0.5	0.05	0.005
Ethylenedibromide[**,1,2]	2A	Organobromide	1000	100	10
Epichlorohydrin[*,1]	2A	Organochloride	500	50	5
Acrylamide[**,1]	2A	Amide	500	50	5
Trichloroethylene[**,1]	2A	[b]CH	5000	500	50
Carbontetrachloride[**,1]	2B	[b]CH	1000	100	10
Cyclophosphamide[**,1]	1	Cytostaticum	50	5	0.5
Benzo[a]fluoranthene[**,1]	2B	[c]PAH	500	50	5
Benzo[a]pyrene[**,1]	1	[c]PAH	500	50	5
Benz[a]anthracene[**,1]	2B	[c]PAH	500	50	5

[a]AH: aromatic hydrocarbons; [b]CH: Chlorinated hydrocarbon; [c]PAH: polyaromatic hydrocarbons; [§]International agency for research on cancer; *Direct acting agent; **Indirect acting agent, 1: DNA adduct forming agent; 2: DNA Cross linking agent

Table S2: Results of the marginal model describing the effect of exposure, i.e., chemicals, dose, and S9, on global DNA methylation in TK6 cells ***in vitro***.

Effect	**Num DF****	**Den DF*****	**F Value**	***p*-Value**
Chemicals	15	67	3.72	<.0001[*]
S9	1	67	21.41	<.0001[*]
Dose	1	67	2.74	0.1024

[*]Significant at α level of 0.05, **Num DF: Numerator Degree of Freedom, ***Den DF: Denominator Degree of Freedom

4. Chapter 4

Adapted from:

Epigenetic Changes in Lymphocytes of Solvent Exposed Individuals

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Abstract

Aims: we investigated global DNA methylation alterations in lymphocytes of solvent workers and chronic toxic encephalopathy (CTE) patients and explored potential gene-environment interactions for glutathione S-transferases.

Study Population: Cross sectional study was setup in 41 referents, 128 solvent workers, and 23 CTE patients.

Results and Discussion: We found a global DNA hypermethylation in solvent-exposed population compared to the referents ($p = 0.001$, $r = -0.544$). Global DNA methylation was negatively associated with exposure. Further, GSTP1 genotypic polymorphism was found to be significantly associated ($p = 0.033$) with global DNA hypomethylation, which describes the potential gene-environment interaction in the etiology of solvent-induced phenotypes.

Conclusion: This study indicates that solvent-induced DNA methylation alterations have an impact on neurotoxicity and development of CTE.

4.1. Introduction

Chronic organic solvent(s) exposure confers risk for human disease (Ziech et al., 2010). Organic solvents comprise chemically heterogeneous compounds, capable of dissolving oils, fats, resins, cellulose acetate and cellulose nitrate, with a widespread application in a range of industries and occupational settings. The volatility and the lipophilicity make organic solvents toxicologically important. Being volatile, organic solvents rapidly contaminate the working environment and pose a major health risk at occupational settings [2]. Exposure to organic solvents mainly occurs through inhalation and skin contact. Organic solvents can damage different tissues e.g., hematopoietic tissue, nervous system and tissues rich in fat content. Due to their lipophilic and hydrophilic properties, solvent exposure affects central nervous system. A specific syndrome, called chronic toxic encephalopathy (CTE) has been known for many years, which results in neurotoxic effects e.g., nausea, dizziness, fatigue, headache, short-term memory loss, attention impairment and personality changes (Arliensoborg et al., 1979, van Valen et al., 2009).

It has been suggested that solvents or reactive metabolites generated after biotransformation of solvents result in long-term health effects. Genetic polymorphisms of enzymes involved in biotransformation might thus play an important role in modifying the response to occupational toxicants. The conversion of solvents to reactive intermediates mainly occurs by oxidation through cytochrome (CYP) P450 family of enzymes. Reactive metabolites are detoxified by conjugation with glutathione, catalysed by glutathione-S-transferase (GST), or by hydrolysis catalysed by microsomal epoxide hydrolase (EPHX1). Kezic et al. (2006) reported an increased risk for the development of CTE in individuals with *CYP2E1*5B* and a decreased risk with the *GSTPE*1C* and *EPHXI* exon 4 genotypes (Kezic et al., 2006). CTE subjects exhibit signs of mild Parkinsonism (Hageman et al., 1999). Genetic polymorphism in dopamine receptor genes was also suggested to have an effect in response to solvent exposure (Noble, 2000). We previously reported that exposure itself increases the risk of CTE, and that genetic polymorphisms in GST i.e., *GSTT1* and *GSTM1* modulates the effect of exposure on neurobehavioral effects. These genetic factors were suggested to have no effect on CTE in the absence of relevant exposure (Godderis et al., 2010). However, it is important to note that genetic factors

alone do not fully describe the phenotype of acquired neurobehavioral disorders. Evidence suggests the dynamic framework of epigenetic regulation may provide answers in the etiologic of phenotypically complex disorder such as CTE.

These findings lead us to investigate the cellular response to solvents exposure through epigenetic pathways. Epigenetics comprises all kinds of structural landmarks on DNA and histones, which code for heritable changes in gene expression without affecting the DNA sequence. Epigenetic information are maintained through post-replication DNA cytosine methylation, post-transcriptional modification of histones and RNA-mediated gene silencing. DNA cytosine methylation is the most studied epigenetic modification. In DNA cytosine methylation, a methyl group is selectively added to the 5' position of the cytosine bases in the DNA sequences rich in CpG islands. In general, CpG methylation is associated with gene silencing (Iacobuzio-Donahue, 2009).

Recently, DNA cytosine methylation is said to provide the missing link between neurodegenerative phenotype and the environment (Iraola-Guzman et al., 2011). A number of environmental and occupational agents, e.g., arsenic, cobalt, dietary factors, alcohol, have been identified to affect the DNA methylation (Fragou et al., 2011). We also reported the global loss of DNA methylation in TK6 (human lymphoblastoid) cells in response to environmental and occupational toxicants (Tabish et al., manuscript submitted). The list of neurobehavioral disorders in which epigenetic factors play an etiopathogenic role is increasing, e.g. Prader-Willi and Angelman syndrome, fragile X (A and E) mental retardation syndrome, autism (Mehler, 2010), schizophrenia (Akbarian, 2010), alcoholism (Ishii et al., 2008), Alzheimer's disease, Parkinson's disease, and Huntington's disease (Urdinguio et al., 2009, Kwok, 2010). Epigenetic deregulation of genome may represent the functional mechanism in neurobehavioral disorders. Based on these observations, we hypothesize the involvement of DNA methylation in the etiopathogenesis of solvent induced neurobehavioral phenotypes such as CTE. In this report, we assessed the global DNA methylation profile of lymphocytes of solvent-exposed workers and CTE patients to investigate the impact of solvent exposure on global DNA methylation. We also looked at the type 2 gene-environment interaction in the context of global DNA methylation.

4.2. Population and methods

The study population, exposure assessment and genotyping methods used in this study have been described in detail elsewhere (Godderis et al., 2010). Study (reference number: B32220072332) was approved by the Commission for Medical Ethics of University Hospital of Leuven (Belgium) and samples were obtained following the informed consent.

4.2.1. Subjects

A cross sectional study was setup with an original population of referents (*n* = 41; non solvent-exposed workers e.g., packagers, drivers, catering staff etc.) from small and medium sized enterprises who were invited to participate voluntarily during their yearly medical examination. Solvent-exposed workers (*n* = 128) with a minimum exposure period of 2 years were selected from a pharmaceutical production plant. They were exposed to mixtures of organic solvents e.g., exposed to chloroform during cleaning of surfaces. CTE patients (*n* = 23) were selected out of a group of 91 CTE patients from the Neuropsychotoxicological Centre of Expertise (Geel, Belgium). CTE patients were included on the condition that the diagnosis for CTE was confirmed in a second independent clinical reference centre of the Belgian fund for occupational diseases.

4.2.2. Exposure assessment

Solvent exposure was assessed by a comprehensive interview to identify the exposure parameters i.e., duration of exposure, and degree of exposure and % work time. For pharmaceutical plant workers, more detailed information was available from historical and current data of air sampling and biomonitoring. Since every participant was not exposed to the same agent, we reported the degree of exposure as a percentage compared to the Belgian threshold limit value (TLV) (e.g., TLV of chloroform = 10 mg/m^3). For each exposure period, the 'degree of exposure * exposure duration * %work time' was determined, which was used to calculate the

cumulative exposure index (CEI). Further details regarding to the exposure assessment are provided elsewhere (Godderis et al., 2010).

4.2.3. Questionnaires

A set of questionnaires regarding to the influencing factors, such as: schooling, lifestyle, medical and occupational histories, possible leisure-time neurotoxic exposures, alcohol consumption, smoking habits, and use of psychotropic medication (anti-psychotics, anti-depressants, mood stabilizers, anti-anxiety agents, etc.) was completed by all participants.

4.2.4. Genotyping

From each participant, a total of 5 ml blood in EDTA tubes was obtained in order to perform genotyping analysis. The *GSTM1* and *GSTT1* polymorphisms were analysed by the method described by Arand et al. (1996), which detects individuals with homozygous deleted genes (Kubota et al., 2012). The *GSTP1* polymorphism in codon 105 was assayed with a PCR-RFLP-based method. For the statistical analysis, each genotype was coded with a binary variable e.g., 1: absent (*GSTM1, GSTT1*) or at least one variant allele (*GSTP1*) and 2: present (*GSTM1, GSTT1*) or wild type (*GSTP1*).

4.2.5. Global DNA methylation analysis

Details of DNA extraction, DNA hydrolysis and global DNA methylation analysis using ultra-pressure liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) have been described elsewhere (Tabish et al., manuscript submitted). In brief, 1µg of extracted DNA per sample was hydrolysed into individual deoxynucleotides and was subjected to LC-ESI-MS/MS to quantify 5-methyl-2'- deoxycytidine (5Me)dC and deoxycytidine (dC). We calculated % global DNA methylation as (5Me)dC/[(5Me)dC+dC] %. The analyses were carried out on a Waters Acquity UPLC equipped with autosampler and a Micromass MS Technologies Quattro Premier mass spectrometer.

4.3. Statistics

Statistical analysis was performed with SPSS v.19.0 (Chicago, USA). Mann-Whitney –U (MW-*U*) test and Kruskal-Wallis statistics were used to compare age, vocabulary and education level, exposure measures, use of alcohol and tobacco and global DNA methylation between groups. Spearman correlations and multivariate linear regression was used to assess the association between global DNA methylation and exposure parameters. Global DNA methylation was determined while correcting for the confounders. Further, we used linear regression models incorporating an interaction term for each GST genotype and exposure parameter to assess the type 2 gene-environment interaction. Relevant population parameters i.e., age, education level, alcohol, and smoking were included in the multiple regression model as independent variables. Significance level (α) was set at 0.05 in all statistical analysis.

4.4. Results

4.4.1. Study population characteristics

Relevant study population characteristics are listed in Table 1, and discussed in detail elsewhere (Godderis et al., 2010). CTE-patients had higher age and longer exposure to solvents than solvent-workers (Table 1). The median CEI was also higher in CTE patients compared to solvent-workers (Table 1).

Table 1: Characteristics of study population.

Characteristic	Referents n = 41 median (25–75 percentile)	Solvent workers n = 128 median (25–75 percentile)	CTE patients n = 23 median (25–75 percentile)
Age (years)	44 (41–50)	41 (35–46)*	52 (49–55)*,**
Education level†	2 (2–3)	3 (3–4)*	2 (2–3)**
Vocabulary level‡	3 (2–4)	4 (3–5)*	3 (3–4)
Alcohol (g alcohol/week)§	60 (10–110)	70 (30–120)	10 (0–40)*,**
Smoking (pack years)	3.84 (0–12)	0 (0–6.6)*	9 (0.5–25)**
Total exposure time (years)	0 (0–0)	4.4 (2.4–10.5)*	24.5 (18–28.5)**
Degree of exposure	0 (0–0)	0.37 (0.18–1)*	1.34 (1.1–1.5)**
Cumulative exposure index (years)¶	0 (0–0)	1.5 (0.3–9.1)*	30.4 (20–36.4)**
Smokers, n (%)	14 (34)	21 (16.4)*	9 (39)**
Use of psychotropic medication, n (%)	3 (7.3)	7 (5.4)	20 (86)*,**

†Level 1: primary school; level 2: lower secondary school; level 3: higher secondary school; level 4: high school; level 5: academic.
‡Level 0: <6 words correct; level 1: 6–8 words correct; level 2: 9–11 words correct; level 3: 12–15 words correct; level 4: 16–17 words correct; level 5: ≥18 words correct.
§One glass of beer or wine = 10 g alcohol.
¶Addition for each job title of 'degree of exposure × exposure duration × percentage of work time'.
**Significantly different compared with referents (Mann–Whitney U-test and χ^2: $p < 0.05$).*
***Significantly different compared with solvent workers (Mann–Whitney U-test and χ^2: $p < 0.05$).*
CTE: Chronic toxic encephalopathy.

4.4.2. Global DNA methylation in solvent exposed and CTE patients

We observed global DNA hypermethylation (M-W *U*, $p < 0.001$) in the lymphocytes of solvent exposed-workers (median global DNA methylation 6.3%, IQR 5.9-6.8%) compared to the referents (median global DNA methylation 4.4%, IQR 4.1-5.2%). CTE patients had a global DNA methylation profile (median global DNA methylation 4.3%, IQR 3.7-4.6%) similar to the referents (M-W *U*, $p = 0.233$) but significantly lower than solvent-workers (M-W *U*, $p < 0.001$).

The association of the global DNA methylation with the different exposure parameters is given in Table 2.

Table 2: Correlations between global DNA methylation and exposure parameters.

Variables correlated to global DNA methylation†	Correlation analysis	Solvent workers (n = 128)	CTE patients (n = 23)	Total exposed group‡ (n = 151)
Without correction				
Total exposure time	Spearman r p-value	-0.123 0.165	-0.025 0.911	-0.435 <0.001
Degree of exposure	Spearman r p-value	-0.121 0.175	-0.185 0.399	-0.342 <0.001
Cumulative exposure index	Spearman r p-value	-0.142 0.110	-0.103 0.639	-0.445 <0.001
Correcting for age				
Total exposure time	Partial r p-value	-0.198 0.025	-0.441 0.040	-0.519 <0.001
Degree of exposure	Partial r p-value	-0.170 0.057	-0.112 0.621	-0.257 <0.001
Cumulative exposure index	Partial r p-value	-0.244 0.006	-0.367 0.093	-0.544 <0.001

Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.
†Global DNA methylation: (5Me)dC/[(5Me)dC+dC]%.
‡Solvent-exposed workers and CTE patients.
CTE: Chronic toxic encephalopathy.

After correcting for age, total exposure time and CEI were significantly correlated with global DNA methylation (solvent-workers: r = -0.198, CTE patients r = -0.441; and solvent-workers: r = -0.244 respectively). Strong association between the exposure and global DNA methylation (all $p < 0.012$) was observed in the multivariate regression models. The association of CEI and global DNA methylation, in the lymphocytes of solvent-exposed and CTE patients, is shown in Figure 1.

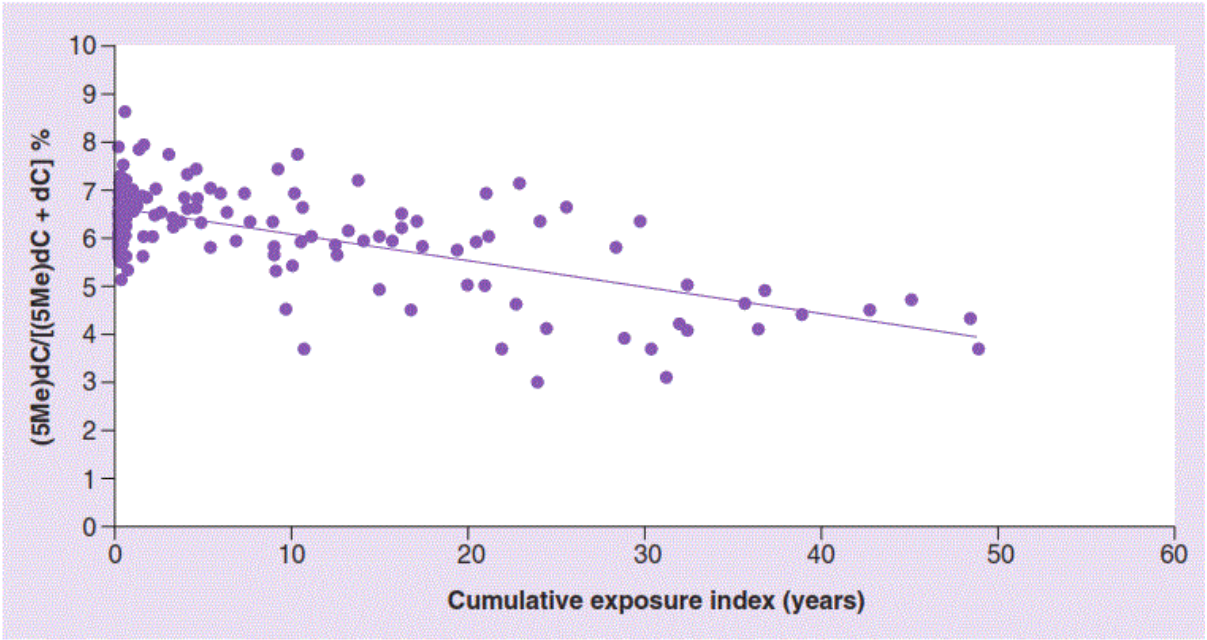

Figure 1: Association of global DNA methylation with cumulative exposure index (years) of total exposed group (n=151). Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.

Age (r = -0.371, p = 0.001), smoking (r = -0.275, p = 0.001) and use of psychiatric drugs (r = -0.425, p = 0.001) were negatively associated with global DNA methylation, while alcohol consumption had positive association with global DNA methylation (r = 0.241, p = 0.001).

4.4.3. Gene-environment paradigm

First, we assessed the global DNA methylation distribution per *GST* genotypes in referents, solvent-workers, and CTE patients group (Table 3). As expected, there was no statistical difference in the global DNA methylation between the 3 genotypes considered (MW-*U*: p>0.05), nor in the total population, or in the respective subpopulations.

Table 3: Global DNA methylation per GST genotypes in referents, solvent workers and chronic toxic encephalopathy patient group.

Genotype		Global DNA methylation†					
		Referents		*Solvent workers*		*CTE patients*	
		Total, n (%)	*Median (25–75 percentile)*	*Total, n (%)*	*Median (25–75 percentile)*	*Total, n (%)*	*Median (25–75 percentile)*
GSTM1	Null	23 (56)	4.7 (4.1–7.0)	71 (55.5)	6.3 (5.9–7.7)	14 (60.9)	4.5 (3.7–5.6)
	Present	18 (44)	4.4 (3.9–6.8)	57 (44.5)	6.4 (6.0–7.3)	9 (39.1)	4.2 (3.9–4.5)
GSTT1	Null	11 (27)	4.2 (4.1–6.8)	29 (2.7)	6.4 (6.3–7.4)	5 (21.7)	4.4 (4.1–4.6)
	Present	30 (73)	4.5 (4.0–7.0)	99 (77.3)	6.3 (5.9–7.7)	18 (78.3)	4.25 (3.7–5.6)
GSTP1	Mutant allele	25 (61)	4.4 (4.1–6.8)	80 (62.5)	6.3 (5.9–7.8)	10 (43.5)	4.5 (4.1–5.6)
	Wild-type	16 (39)	4.8 (4.1–7.0)	48 (37.5)	6.4 (5.9–7.4)	13 (56.5)	4.1(3.7–5.0)

†Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.
CTE: Chronic toxic encephalopathy.

Further, we assessed the association of type 2 gene-environment interaction with global DNA methylation. We included an interaction term for each *GST* genotype and relevant exposure parameter in the regression models. Degree of exposure was excluded from further analysis because it was not significantly associated with global DNA methylation. Out of the three *GST* polymorphisms included in this study, only *GSTP1* polymorphism was found to be significantly associated with global DNA hypomethylation (p = 0.033) in response to total exposure time in the entire study

population (Figure 2 and Table 4). We found a borderline *GSTM1*–exposure time interaction with global DNA hypermethylation (p = 0.053) in solvent-workers (Table 4).

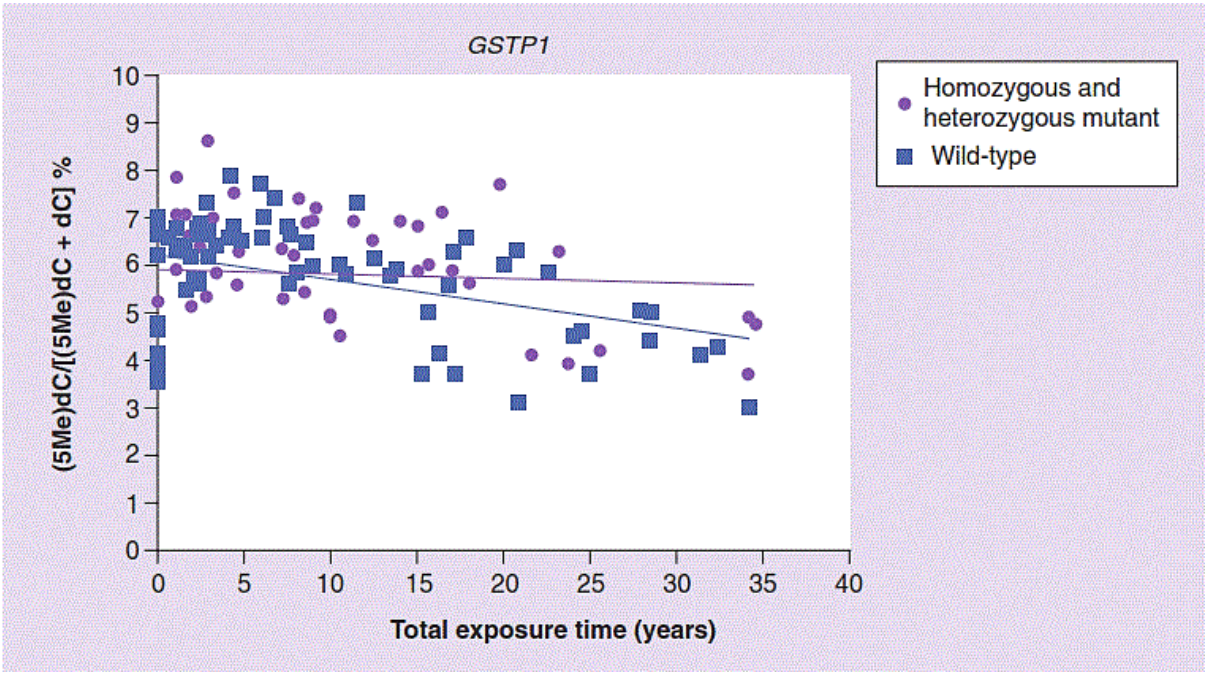

Figure 2: Effect of GSTP1 genotypic variation on global DNA methylation profile in the lymphocytes of total study population (n=192) with total exposure time. Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome. R^2 linear of homozygous and heterozygous mutant individuals = 0.006; R^2 linear of wild-type individuals = 0.161.

Table 4: Genotypic influence on global DNA methylation with total exposure time as an independent variable in the solvent exposed and total population.

Dependent	Independent variable	B	SE	β	Partial r	p-value	Adjusted R^2
Global DNA methylation†,‡	Constant	6.037	0.261			<0.001	0.061
	Total exposure time	0.033	0.031	0.314	0.095	0.290	
	GSTM1§	0.362	0.175	0.276	0.183	0.041	
	GSTM1 × total exposure time	-0.036	0.019	-0.648	-0.173	0.053	
Global DNA methylation†,¶	Constant	5.645	0.321			<0.001	0.059
	Total exposure time	0.029	0.029	0.225	0.074	0.309	
	GSTP1#	0.267	0.221	0.114	0.088	0.227	
	GSTP1 × total exposure time	-0.040	0.018	-0.506	-0.155	0.033	

Global DNA methylation is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome.
†*Global DNA methylation = (5Me)dC/[(5Me)dC+dC]%.*
‡*Solvent-exposed population (n = 151).*
§GSTM1 *genotype (variable 1 = null, variable 2 = wild-type).*
¶*Total population (n = 192).*
#GSTP1 *genotype (variable 1 = homozygous or heterozygous mutant; variable 2 = wild-type).*
SE: Standard error.

4.5. Discussion

Workers chronically exposed to solvents are at risk of developing CTE. Epigenetic mechanisms, such as DNA methylation, might play a role in the development and progression of CTE. In this study, we investigated whether chronic solvent exposure results in altered epigenetic status, which could lead to a long term reprogramming of genetic information with subsequent development of CTE. Duration of solvent exposure was negatively associated with global DNA methylation while intensity of exposure was only significantly associated with global DNA hypomethylation when considering all exposed individuals. These finding could be suggestive for an age-dependent effect, known to lead to a gradual demethylation of DNA (Christensen et al., 2009). Nevertheless, even after controlling for age we could confirm these results, observing a strong association between global DNA methylation and CEI (Figure 1, Table 2). Our results suggest that epigenetic changes may have an impact on the development of CTE. This is in line with findings in other neurodegenerative and neuropsychiatric diseases and in neurotoxicity induced by ethanol and drugs (Fragou et al., 2011). DNA hypermethylation has been proposed to play a part in the pathogenesis of schizophrenia (Mill et al., 2008, Zhubi et al., 2009). DNMT1expression alterations in GABAergic neurons are also reported to contribute in the occurrence of schizophrenia symptoms (Mill et al., 2008, Zhubi et al., 2009).

Studies have described the induction of both DNA hypo- and hypermethylation in response to solvents, and environmental and occupational carcinogens (Zhong and Mass, 2001). The outcome seems to depend on the type of agent studied and the exposure characteristics. For example, low level exposure to benzene has shown to induce DNA hypomethylation of repetitive DNA elements as well as hypo- and hypermethylation of specific genes in peripheral blood DNA (Bollati et al., 2007). In a recent human cohort based study, the PAH exposure has been linked with global DNA hypermethylation (*LINE-1* and *Alu* repetitive elements). Conversely, DNA hypomethylation of specific promoter sequences (*p15* and *HIC1*) was suggested in PAH-exposed workers (Herbstman et al., 2009, Pavanello et al., 2009). Global loss of DNA methylation *in vitro* in response to solvent exposure (chronic formaldehyde) has also been suggested in a recent report (Liu et al., 2011). The mechanisms by which

solvents exposure induces DNA methylation alterations are still unclear. Solvents have been shown to induce higher levels of oxidative stress (Dreiem et al., 2005). Oxidative stress affects DNA methylation by changing the S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) ratio through regulating the methionine metabolic pathway in human lung epithelial-like (A549) cells (Ziech et al., 2011, Panayiotidis et al., 2009). Decreased level of methyl donor SAM and DNA methyltransferase 3B (DNMT3B) expression due to alcohol exposure affected the DNA methylation in hepatic tissue (Szyf, 2011). Oxidative stress, DNA methyltransferases expression and DNA cross-links and strand breaks in response to benzene and formaldehyde exposure are suggested underlying mechanisms which disrupt genomic methylation signatures (Liu et al., 2011, Bollati et al., 2007, Szyf, 2011).

Induction of global DNA hypermethylation was observed in solvent exposed workers compared to the CTE patients. A number of factors could explain the observed difference in global DNA methylation profiles in these groups. Solvent-exposed workers were still exposed to solvents while CTE patients were no longer exposed but rather had a past history of chronic solvent exposure. Alcohol consumption was positively associated with global DNA hypermethylation in this study. Alcohol consumption was higher in the solvent-exposed workers than in the CTE group. These factors could explain the dynamics of DNA methylation differences observed in the lymphocytes of solvent-exposed workers and CTE patients. On the other hand, sometimes results are contradictive. For example, both the induction of hypo- and hypermethylation of specific genomic regions have been described in response to alcohol exposure (Ishii et al., 2008, Liu et al., 2009).

In this study we used peripheral blood lymphocytes, which are heterogeneous population of cells with a short life span compared to the neurons that represent the target tissue in CTE, to profile the methylome. The question remains whether lymphocytes can be used as surrogate tissue. Lymphocytes of solvent-workers show DNA methylation stress because they are currently exposed to the solvents, whereas absence of current solvent exposure in CTE patients could result in the alleviation of sustained DNA methylation stress in lymphocytes, leading to a DNA methylation pattern similar to the controls. Since neurons are terminally divided cells, and it is to

be expected that epigenetic changes in the neuronal cells be maintained in CTE individuals with previous history of chronic solvent exposure. Studies have reported similar DNMT1 and DNMT3a expression profiles in peripheral blood lymphocytes and telencephalic GABAergic neurons of schizophrenia patients, which supports the popular concept that readily available blood lymphocytes can express epigenetic markers relevant to schizophrenia (Zhubi et al., 2009). This suggests that lymphocytes could be used as reporter cells to assess the risk factors of developing neurobehavioral disorders. Additionally, lymphocytes provide a live cell model to examine the functional consequence of epigenomic alteration in solvent-exposed population and CTE patients, as opposed to post-mortem brain where mRNA and DNA could get degraded. Epidemiological studies have also reported the association between global DNA methylation in white blood cells and demographic, environmental, behavioural risk factors and cancer (Terry et al., 2011). Studies investigating the association between DNA methylation in white blood cells and different disease endpoints are intriguing but need to be replicated in larger and prospective studies.

Although the genetic vulnerability towards neurobehavioral disorder has been described in many reports, there exists an ample room for epigenetic explanations for risk factors in developing neurobehavioral disorders. Genetic polymorphisms in metabolizing enzymes and dopamine receptor genes, have been described as conferring advantageous and disadvantageous towards the development of CTE (Godderis et al., 2010). However, phenotypic plasticity is more than a simple property of genetic variation in a number of genes. Genetic differences could affect the cellular epigenetic status in response to environmental factors. The current study also highlights the involvement of a gene-environment interaction that defines the individual's susceptibility towards solvent exposure. There was no statistical difference in the global DNA methylation between the 3 genotypes considered (MW-*U*: $p>0.05$), nor in the total population, or in the respective subpopulations. This is to be expected since we focused on genes involved in the metabolism. Our results suggest significant *GSTP1*–total exposure time interaction with global DNA hypomethylation for the entire study group. This implies the involvement of type 2 gene-environment interaction. *GSTP1* polymorphism does not predetermine for neurotoxic effects in absence of solvent exposure. However, in case of exposure, *GSTP1*–exposure time interaction modulates the epigenetic response, and may

confers individual's susceptibility towards neurotoxic effects. Epigenetic processes themselves increases individual's susceptibility towards solvent exposure as discussed previously. The *GSTM1* genotype showed a borderline association with global DNA methylation in solvent workers. This is in line with our previous study, indicating that the *GSTM1* polymorphism affects an individual's susceptibility towards CTE (Godderis et al., 2010).

Certain methodological limitation specific to this study should be considered. The blood samples, to obtain the genome DNA, were collected over a period of six months. The time-laps in sample collection among participants could have an effect on DNA methylation process. After sample collection, DNA was extracted and store at -80°C until further analysis. Also the gene-specific DNA methylation status was not assessed in this study. In this regard, it is possible that genetic factors, which are important in aetiology CTE, are differentially methylated in response to solvent exposure. Another limitation of this study is the selection bias, which could effect the exposure assessment, and could also have an affect the global DNA methylation measurement. Since it is a cross-sectional study, it is not possible to address the issue of reverse causality in the current study design.

In conclusion, we described the global DNA methylation alterations in solvent-exposed workers and CTE patients with a history of chronic solvent exposure. We also delineate the potential association of genetic difference in drug metabolizing enzyme i.e., *GST* with global DNA methylation in these groups. In future studies, we will focus on the effects of solvent exposure on gene specific promoter methylation e.g., dopamine receptor genes, promoter sequences of drug metabolizing enzymes, in solvent-exposed and CTE patients. We will also characterize the role of epigenetics in gene-environment-interaction model towards the development of CTE-related phenotypes.

Future Prospective

Epigenetics is an emerging area of the genome in disease research. Molecular and cellular networks regulating the tissue specific epigenetic information could help understanding the basis of neurobehavioral disorder such as CTE. Since the first application of epigenetic information in clinical setting to diagnose two imprinted disorders (Parader-Willi and Angelman Syndrome) based on gene-specific differential

methylation, several methodological advancements e.g., genome-wide methylation analysis based on next-generation DNA sequencing, have been made in the field of epigenetic (Kubota et al., 2012). These genome-wide assays will help scientific community to dissect both genetic and epigenetic factors in the aetiology of acquired neurobehavioral disorders. Environmental exposure induces DNA methylation alteration in the cells. However, the extent and duration of the methylation alterations to confer disease phenotype remains to be understood fully.

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5. Chapter 5

Adopted from:

DNA methylation changes by exposure to nanomaterial in mice blood and lung

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Abstract

DNA methylation effects, including global DNA methylation/hydroxymethylation and gene specific methylation, associated with exposure to nanomaterial (NM) are largely unknown. In this study, we investigated the effects of NM exposure on DNA methylation. We exposed BALB/c mice to gold NPs (AuNPs) of 5nm, 60nm and 250nm diameter; single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) at high dose of 2.5 mg/kg and low dose of 0.25 mg/kg for 48 hrs. Cytotoxicity assays revealed AuNPs induced macrophage-dominant while CNTs induced macrophage and neutrophil-dominant immune response. In exposed mice, no effects of AuNPs and CNTs exposure were observed on oxidative stress and DNA damage, global DNA methylation and hydroxymethylation. In mice lung tissue, following DNA methylation effects were observed; AuNPs 60nm exposure induced promoter CpGs hypermethylation in *Atm*, *Cdk* and *Gsr* genes, while promoter CpGs hypomethylation in *Gpx* gene; changes in promoter methylation of *Gsr* and *Trp53* was also observed between low and high dose of AuNPs 60 nm and AuNPs 250 nm respectively; AuNPs size effects on promoter methylation was observed for *Trp53* gene; and CNTs exposure affected the promoter methylation of *Atm* gene. In mice blood DNA, the only effect observed was the induction of promoter hypermethylation in *Pparg* gene by exposure of AuNPs 60 nm high dose compared to the AuNPs 60 nm low dose. Epigenetically altered genes were involved DNA apoptotic process, immune system process, metabolic process and response to stimulus pathways. In conclusion our results showed that exposure to NM lead to gene specific methylation changes in vivo.

5.1. Introduction

Humans are exposed to airborne ultra-fine particles (UFP) from different sources (Terzano et al., 2010). This exposure has changed because of the anthropogenic factors, and more recently with the rapid developments in nanotechnology, which is engineering nanomaterial (NM) with size-dependent properties called nanoparticles (NPs). NPs are defined as particles with at least one dimension less than 100 nm (Oberdorster et al., 2005). Nanomaterial e.g., gold nanoparticle (AuNPs), titanium dioxide NPs, zinc oxide NPs, and single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCTs) are currently used in many applications. For example, some of them are used chemotherapy, targeted drug delivery systems (TDDS), intracellular gene regulation, as well as in aerospace, automobiles, electronic and optical applications (Allhoff et al., 2009). Their widespread presence, small size and unique physicochemical properties also pose public health concerns (Luyts et al., 2013). Human exposure to NPs mainly occurs via inhalation, skin contact and ingestion. NPs exposure via inhalation is particular important as NPs can penetrate deep into the pulmonary interstitium, and can translocate from lungs into systemic circulation (Choi et al., 2010). In line with this, it is important to investigate the effects of nanomaterial in lungs and in blood cells. Also, in TDDS, AuNPs and CNTs are introduced into the body, which raises question on the fate and effects of these nanomaterials in the body.

Gold (Au) is considered relatively inert and biocompatible; however, recent studies raised concern of the biocompatibility of Au in nano-size range (Alkilany and Murphy, 2010). Also, physicochemical properties of CNTs pose health concerns similar to that observed with asbestos, such as development of mesothelioma (Boczkowski and Lanone, 2012). Different mechanisms have been proposed for the observed toxicity of AuNPs and CNTs e.g., induction of oxidative stress, DNA damage, immune deregulation etc (Manke et al., 2013). In line with these observations, it is shown that exposure to some NPs alters the expression of genes implicated in exposure-induced pathways (Rahman et al., 2009, Kim et al., 2009). AuNPs exposure has shown to alter the expression *IL-6, TNF-α, CDK* genes (Khan et al., 2013). Carbon nanotubes exposure has also been associated with changes in

expression of genes such as *C-MYC*, *GSR*, *IL6*, *Gpx3* and many other genes regulating diverse cellular pathways (Alazzam et al., 2010, Pacurari et al., 2011). This indicates that NM exposure alters cell signaling pathways that regulate gene expression.

Epigenetic modifications are involved in regulating gene expression. Epigenetic modifications i.e., DNA methylation, histone modification, microRNAs, alter gene activity without altering the DNA sequence. DNA methylation (5-Methylcytosine: 5mC) is one of the most studied epigenetic modifications (Jones and Baylin, 2002) occurs almost exclusively on cytosine followed by guanine base (i.e., CpG dinucleotides) in humans. Within genome, regions of high density of CpG dinucleotides are present called CpG islands (CGIs). CGIs are preferentially distributed within the gene promoter regions where they regulate gene expression (Esteller, 2002). Several classes of environmental chemicals, including metals, particulate matter, and endocrine/reproductive disrupters are shown to modify gene promoter methylation marks (Baccarelli and Bollati, 2009). Moreover, xenobiotic exposure is also shown to affect global DNA methylation (total number of methylated cytosines in the genome), and global DNA hydroxymethylation (5-hydroxymethylcytosine: 5hmC) (total number of hydroxymethylated cytosines in the genome) (Dao et al., 2014, Tabish et al., 2012). 5hmC is a DNA cytosine modification (hydroxymethylation of cytosines), which recently gained interest because it represents the DNA demethylation pathway (Tabish et al., 2012). In this study, we investigated the DNA methylation effects, including global 5mC, 5hmC and gene specific methylation, induced by exposure of NM in BALB/c mice.

5.2.Methods

5.2.1. Gold nanoparticles and carbon nanotubes

Citrate coated colloidal AuNPs of three primary sizes (small sized AuNPs: 5nm; medium sized AuNPs: 60 nm, and large sized AuNPs: 250 nm) were obtained from BBInternational (Cardiff, UK). SWCNTs (Raw Soot) were purchased from National Institute of Standards and Technology (NIST) (SRM: standard reference materials;

2483) (Gaithersburg, US). AuNPs were thoroughly characterized for their primary particle diameter, hydrodynamic diameter and zeta-potential in baxter water (B.Braun Medical Inc, Irvine, US) and in 2% serum by dynamic light scatting (DLS) method. MWCNTs (NM-400) were obtained from European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (Milan, Italy). CNTs were characterized for their size distribution in H_2O by electron microscopy (Powers et al., 2007).

5.2.2. Preparation of nanomaterial

Stock suspensions (2 mg/ml) of powder samples (SWCNTs & MWCNTs) were prepared in pure H_2O with 2% mouse serum by ultra-sonication (PTS Technics, Huddinge, Sweden) for 16 minutes. NPs stock suspensions were used within an hour of preparation. Working concentration (high dose: 1mg/ml; low dose: 100 µg/ml) of CNTs and AuNPs were prepared prior to instillation in saline solution with 0.2% mouse serum in lipopolysaccharide (LPS) free vials.

5.2.3. Animals

Male BALB/c mice (~20g, 7 weeks old) were obtained from Harlan (Horst, The Netherlands), and housed in filter cages in a conventional animal house at controlled temperature (21 ± 1°C) and humidity (50 ± 10%) with 12-h dark/light cycles. Mice were fed on lightly acidified water and pelleted food (Trouw Nutrition, Ghent, Belgium) *ad libitum*. All experimental procedures were approved by the Local Ethical Committee (Katholieke Universiteit Leuven, Leuven, Belgium) with the project number ML8557.

5.2.4. Experimental design

Mice were divided into five experimental groups designated as; 1) shame-control (n = 8), 2) vehicle-control (n = 8), 3) decitabine group (n = 5) which was used as a positive control, 4) AuNPs (n = 5/group): 5nm AuNPs low dose (0.25 mg/kg) and high dose

(2.5 mg/kg), 60 nm AuNPs low dose (0.25 mg/kg) and high dose (2.5 mg/kg), and 250 nm AuNPs low dose (0.25 mg/kg) and high dose (2.5 mg/kg), 5) carbon nanotubes (*n* = 5/group); SWCNT low dose (0.25 mg/kg) and high dose (2.5 mg/kg) and MWCNTs low dose (0.25 mg/kg) and high dose (2.5 mg/kg).

Mice were anesthetized in the chamber using isoflurane (3-5%) (Abbott Laboratories, SA Abbott NV, Ottignies, Belgium) for 2 minutes. Each mouse received 50 µl of working NPs solution or decitabine, (1mg/kg prepared in saline with 0.2% mouse serum) or vehicle (saline with 0.2% serum) by single intra-tracheal instillation by 1 ml syringes (BD, Erembodegem, Belgium) followed by 200 µl of air. Sham-control mice were also anesthetized and instilled with 250 µl of air. Mice were weighted before instillation and examined after instillation till fully recovered from the anesthesia or any adverse effects e.g., anxiety etc. After instillation mice were transferred to the animal facility for 48h. After 48h of exposure, mice were weighed; sacrificed using an overdose of pentobarbital (90 mg/kg *i.p.*), blood was sampled from the retro-orbital plexus in K_3EDTA coated vials and flash-frozen in liquid nitrogen. Mice lungs were perfused with saline solution to clear them from blood cells. Mice lungs were dissected into each lobe, transferred into sterile vials, weighed and samples were flash-frozen. Samples were transferred to -80 °C till further analysis.

5.2.5. Bronchoalveolar lavage and bronchoalveolar lavage fluid processing

Bronchoalveolar lavage (BAL) fluid was sampled and cytospin slides were prepared as described previously (De Vooght et al., 2009). BAL cells were stained with trypan blue dye (Invitrogen, Belgium) and total cell count (TCC) BAL cells was performed under the light microscope. BAL cells were fixed on slides, stained with hematoxylin and eosin and differential counts of BAL cells were performed. Images of BAL cells were taken with a zeiss axiovert 220M microscope equipped with axiovision rel. 4.8.2 imaging software at a 100x oil lens with a 10x ocular lens.

5.2.6. Lung cytokine measurements by cytometric bead array

Cytokines (Il-1, Il-4, Il-5, Il-6, Il17a, KC) expression in mice lung tissue was analyzed by cytometric bead array kits (CAB, BD biosciences, Belgium) as per manufacturers protocol on LSR Fortessa flow cytometry platform equipped with FCAP array v3.0 software (BD biosciences, Belgium). Briefly, lung tissue was homogenized in lysis buffer and protein concentration of the lysates was measured. Cytokine concentrations were calculated based on standard curve data using FCAP Array software (BD Biosciences).

5.2.7. DNA damage measurement by comet assay

The comet assay was conducted in accordance with the standard protocol "European Network on the Health and Environmental Impact of Nanomaterials" issued by the ENPRA(risk assessment of engineered nanoparticles) project. All experimental processing for the comet assay was done at 4 ºC in dark. Comet assay consumables were purchased from (Trevigen Inc, Gaithersburg, US). BAL fluid was centrifuged at 2000xg for 10 minutes. Cell pellet was resuspended in 250 µl of saline solution, and 5 µl of this solution mixed with LM agarose were applied on the comet slides. LM agarose was allowed to set for 10 minutes and slides were immersed in the cell lysis solution for 1h. Further, slides were immersed in alkaline unwinding solution (30 minutes) prior to electrophoresis at alkaline condition (pH > 13) for 30 minutes at constant voltages. Slides were washed in H_2O, dehydrated in 70% ethanol for 5 minutes, dried, stained with cyber green in TE buffer, and slides were imaged on fluorescence microscope (Olympus Corporation, Tokyo, Japan). Images were analyzed using autocomet software (TriTek Corp, Sumerduck, US).

5.2.8. Oxidative stress measurement by Liquid chromatography-mass spectrometry

Lung samples were minced immersed in 1 mM N-ethylmaleimide (NEM); a blocking thiol agent to prevent rapid oxidation of GSH. The measurements of the reduced (GSH) and oxidized (glutathione disulfide, GSSG) forms of glutathione were performed by liquid chromatography-mass spectrometry (LC-MS) in-house developed method, partially based on the method of Guan et al., 2003 (Napierska et

al., 2012). The LC-MS analysis was conducted on a Waters Acquity UPLC coupled to a Micromass MS Technologies Quattro Premier mass spectrometer using electron spray ionization (ESI). The LC separation was done on a Waters Acquity UPLC BEH C18, 50 mm × 2.1 mm, 1.7 µm column, held at a temperature of 40 °C.

5.2.9. Global DNA methylation measurement by LC-MS/MS

5-methylcytosine (5mC) and hydroxymethylation (5hmC) in mice lung DNA samples (DNA isolated using Qiagen Blood and Tissue kits, Qiagen; Venlo, Netherlands) were performed as described previously (Tabish et al., 2012, Lode Godderis, 2014). One µg of DNA was hydrolyzed into individual nucleosides, and samples were subject to LC-MS analysis to quantify the absolute amount of 5mC and 5hmC in control and exposed lung DNA samples. Global 5mC and 5hmC analysis was performed in mouse lung DNA samples only because not enough DNA was obtained from mouse blood samples to carry out global 5mC and 5hmC analysis. 5mC is expressed as a percentage of 5-methylcytosine versus the total number of cytosines present in the genome. 5hmC is expressed as percentage of 5-hydroxymethylcytosine versus the total number of cytosines present in the genome.

5.2.10. Gene specific DNA methylation measurements by bisulfite pyrosequencing

Based on literature, we selected cellular pathways often responsive to environmental exposure e.g., oxidative stress pathway, immune pathway, cell cycle regulation pathways, DNA methylation pathway etc. Within these pathways, we selected nineteen (n=19; supplementary table T1) candidate genes with reported gene promoter alterations in response to xenobiotic exposure. For the selected genes, we designed bisulfite-PCR pyrosequencing assays (n=17) in order to investigate if the NPs exposure alters their gene promoter methylation (supplementary table T1). Genomic DNA was treated with the EZ DNA methylation Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Final elution volume was 40 µl with M-elution buffer. PCR was carried in 30 µl using 15 µl of GoTaq Green Master mix (Promega), 10 pmol forward and 10 pmol reverse primers, 50 ng of

bisulfite-treated DNA, and water to reach 30 µl final volume. Amplicons were analyzed in 2% agarose gel. Pyrosequencing was performed as previously described using 0.3 µm sequencing primer (Ali M. Tabish, 2014). PCR and sequencing assays information are given in supplementary table T2. We designed control oligo for 100% DNA methylation (PSQ-C oligo), 0% DNA methylation (PSQ-T oligo) and the sequencing primer for control oligo. We mixed PSQ-C oligo (or PSQ-T oligo) with sequencing oligo in PyroMark Annealing Buffer (QIAGEN Inc., Valencia, CA) and performed pyrosequencing (sequencing entry C/TGTAT) (supplementary table T2). The methylation level is expressed using % 5mC.

5.3.Statistical analysis

Effects of NM exposure on immune endpoints (BAL total cell and differential cell count, cytokine measurements), DNA damage, global DNA methylation and hydroxymethylation were assessed using Wilcoxon test with dunn all pairs post-hoc analysis using JMP v. 10 (SAS institute, NC, USA) .

In order to perform statistical analysis on the results of gene promoter methylation, we took a three-step approach. In the first step, we took an average of methylation of all CpGs analyzed for a given gene. Wilcoxon test was performed taking average methylation of all CpGs per gene as dependent variable and exposure groups as independent variables. Wilcoxon test returned *p*-value of test statistics. In the second step, we performed statistical analysis per CpG methylation. In this analysis, Wicoxon test was performed taking each CpG methylation within a gene (e.g., Atm CpG#1) as dependent variable and exposure groups as independent variables. *P*-values of test statistics were returned by Wicoxon test. In the third step, we performed dunn all pair post hoc analysis on methylation values of exposure groups where Wilcoxon statistics returned significant exposure effects. Dunn all pair post-hoc analysis was performed in order to investigate exposure parameters i.e., AuNPs size and exposure dose (Figure 3, Table 3), CNTs shape and exposure dose (Figure 4, Table 3), responsible for the observed variance in methylation in lung and blood DNA. α level of 0.05 defined as significant. Graphs were made using SPSS software (SPSS Inc., Chicago, IL).

5.4. Results

5.4.1. Nanomaterial characteristics

Physicochemical characteristics of NM used in this study where thoroughly characterized for their size and charge distribution (supplementary table T3 and T4). AuNPs average diameter Average diameter of AuNPs measured with DLS was greater in 2 % serum than their average diameter in H_2O. Distribution curves (supplementary figure F1) for AuNPs show the aggregation of each NP. Curves show the particle aggregation decreases with increase in particles size in 2 % serum compared to their aggregation in H_2O. AuNPs showed negative zeta potential in H_2O and in 2 % serum. In H_2O, AuNPs showed size dependent increase in negative zeta potential, while in serum this dependency was not observed. These charges stabilize the suspension via repulsive forces.

5.4.2. Cytotoxicity of nanomaterial

In order to examine the cytotoxicity of NM exposure, we examined total and differential changes in BAL cells count. We observed significantly higher total cell count in response to AuNPs and CNTs exposure compared to the non-exposed mice (Figure 1a). BAL differential cell count revealed macrophage and neutrophils dominant influx into lung interstices after AuNPs and CNTs exposure respectively compared to control mice (Figure 1b). Percent BAL cells that had taken up/or were associated with the AuNPs and CNTs are shown in figure 1c. In sham- and vehicle control groups no macrophages were observed that had taken up NPs; therefore, they are not visible in figure 1c. Images of BAL macrophages in AuNPs and CNTs exposed and control mice showed that AuNPs and CNTs were taken up/ or associated with the BAL cells in a dose response manner (Figure 1d-i). We did not observe changes in selected cytokines in mice lung in AuNPs and CNTs exposed mice compared to the controls (supplementary Figure F2).

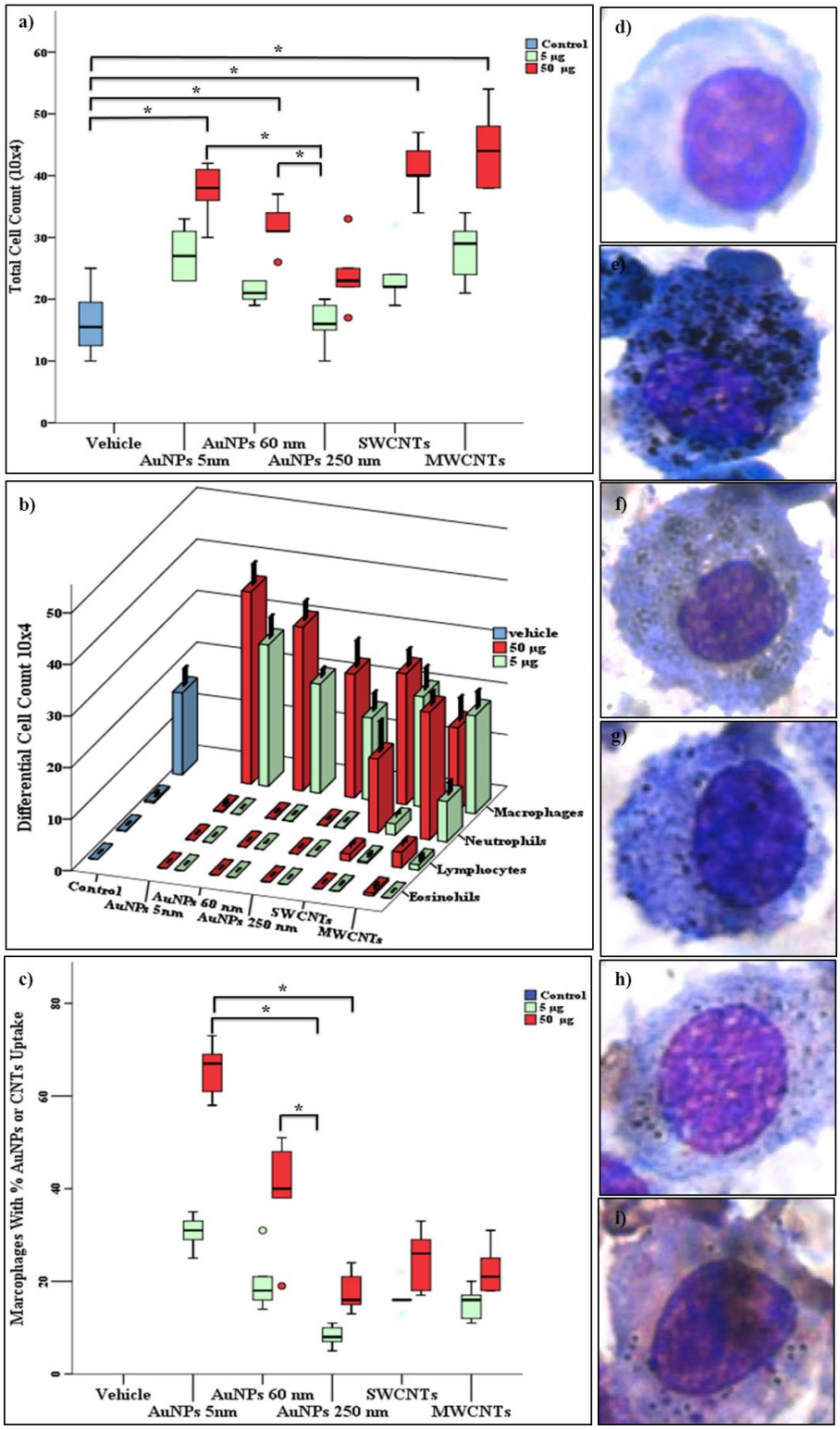

Figure 1 a-i: Bronchoalveolar lavage (BAL) analysis. a): total cell count; b): differential cell count.; c): uptake/association by/with BAL macrophages. For clarity of presentation in panel b, significant groups are not annotated. In panel b, macrophages count was significant in following exposure groups: AuNPs 5nm 50µg and AuNPs 60nm 50µg compared to the vehicle; AuNP 5nm 50µg compared to AuNP 250nm 5µg; and AuNPs 60 nm 50µg compared to the AuNP 250nm 5µg dose categories. Neutrophils count was significant in following exposure groups: SWCNT 50µg and MWCNTs 50µg

compared to vehicle. Lymphocytes count was significant in following exposure groups: SWCNT 50µg and MWCNTs 50µg compared to vehicle. Representative images of macrophage d): vehicle; e): AuNPs 5nm; f): AuNPs 60nm; g): AuNPs 250 nm; h): SWCNTs; i): MWCNTs., In panel a and c; the box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles outside the ends of whiskers. Data in panel b is represented as median ±SD. Asterisk sign (*) shows significance levels at p=0.05. Gold nanoparticles: AuNPs; single-walled- and multi-walled carbon nanotubes: SWCNTs and MWCNTs.

5.4.3. Oxidative stress and DNA damage effects

NPs exposed mice did not show induction of oxidative stress, or DNA damage compared to the control mice (supplementary figure F3 and F4 respectively).

5.4.4. Global DNA methylation and hydroxymethylation in lungs

Wilcoxon statistics showed no significant effects of AuNPs exposure on 5mC and 5hmC levels. Also no effects of CNTs exposure were observed on 5mC and 5hmC levels compared to the controls (Figure 2a-b).

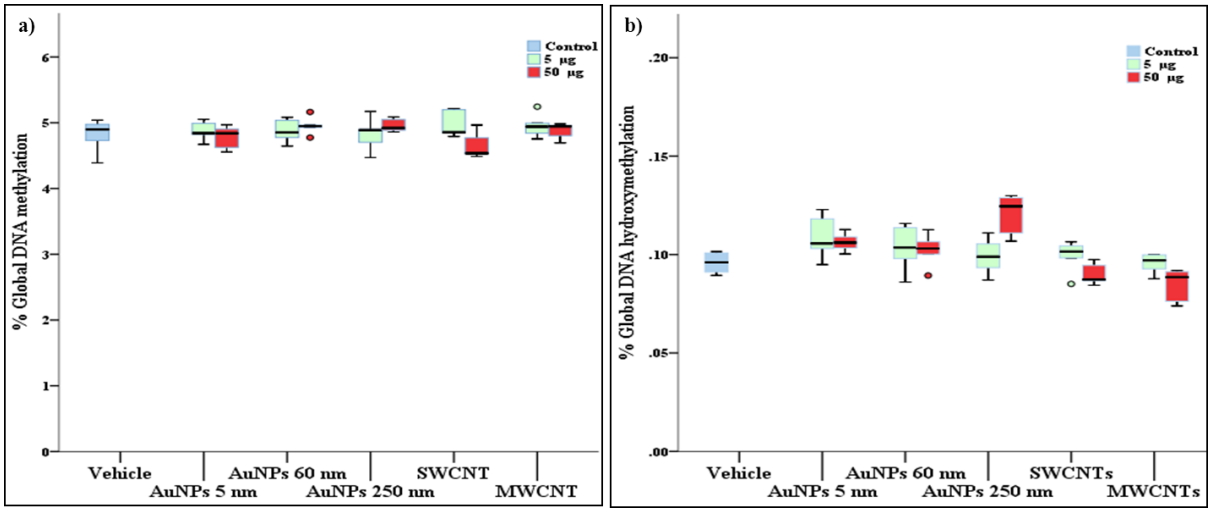

Figure 2: Global DNA methylation (5mC) and hydroxymethylation (5hmC) in lungs. a): no significant effects (Wilcoxon test) of gold nanoparticle (AuNPs) and single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) were observed on 5mC (p=0.667 and 0.284 respectively). b): also no significant effect of AuNPs exposure on lung 5hmC were observed (p=0.107). However, CNTs exposure showed significant effect on 5hmC (p=0.024) levels by Wilcoxon statistics, while no group remained significant after multiple comparisons (Dunn all pairs post-hoc). In panel a and b; box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles outside the ends of whiskers.

5.4.5. Gene promoter methylation in lungs and blood

Effects of NMs exposure on average gene promoter methylation and promoter CpG methylation were investigated: in lung tissue of mice exposed to AuNPs (supplementary table T5-a); in lung tissue exposed to carbon nanotubes (supplementary table T5-b). The effects of NMs exposure on average gene promoter methylation and promoter CpG methylation were also profiled: in blood tissue of mice exposed to AuNPs (supplementary table T6-a); in blood tissue exposed to carbon nanotubes (supplementary table T6-b). *P*-values significant at preset alpha level of 0.05 are highlighted in red, while *p*-values with borderline significance compared to the preset alpha level of 0.05 are highlighted in yellow (supplementary tables 5 a-b and 6 a-b).

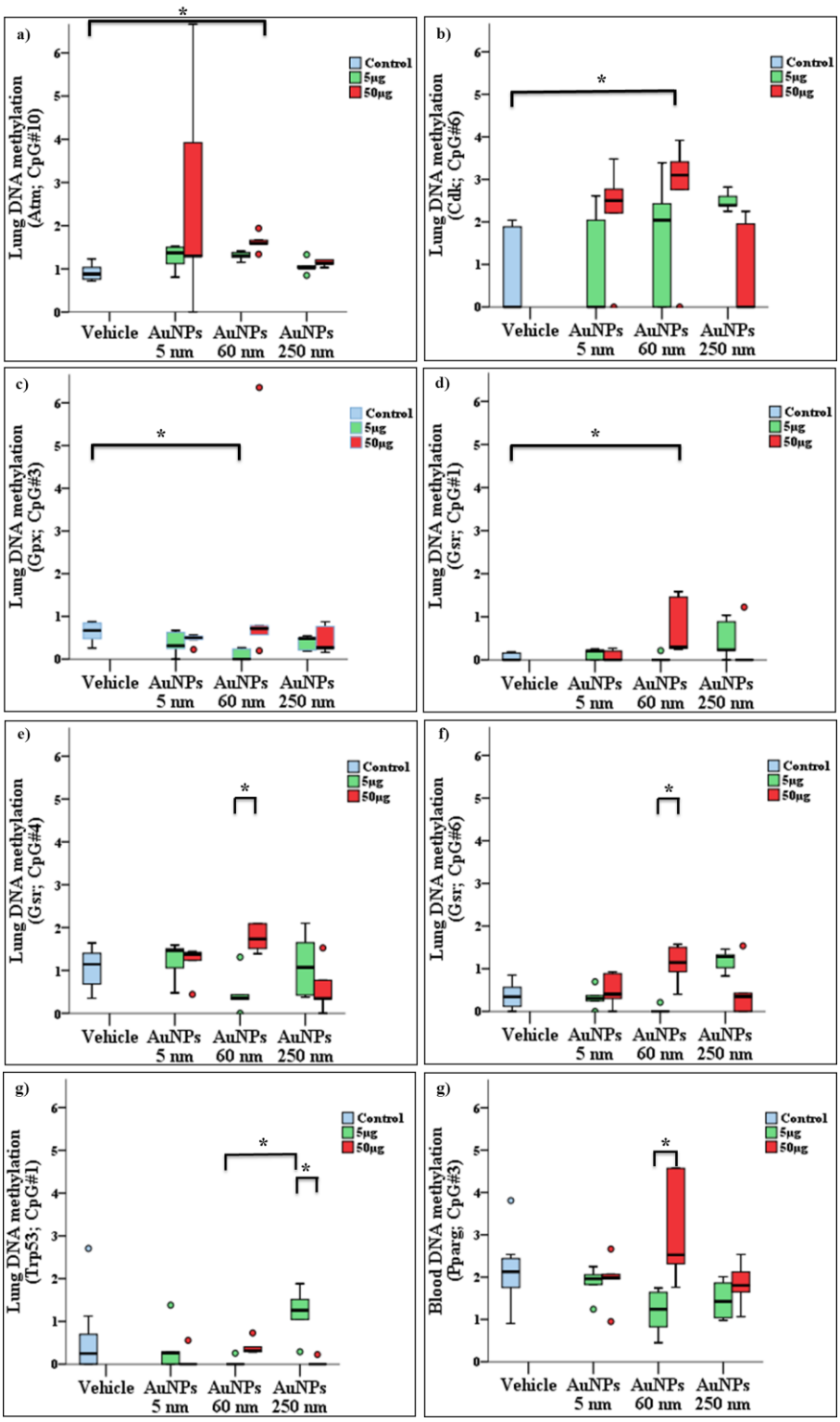

Figure 3: Effect of gold nanoparticles (AuNPs) exposure on gene promoter methylation. Bars connect exposure groups with significant methylation difference a-g): effects of AuNPs exposure on promoter methylation levels of *Atm*(a), *Cdk*(b), *Gpx*(c), *Gsr*(d-f), and *Trp53*(g) genes in lungs; h): effect of AuNPs exposure on gene promoter methylation levels of *Pparg* gene in blood. In panels, box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles outside the ends of whiskers. Asterisk sign (*) shows significance levels at p=0.5. *Atm*: ataxia telangiectasia mutated; Cdk; cyclin-dependent kinase; *Gsr*: glutathione reductase; *Gpx*: glutathione peroxidase; *Trp53*: tumor protein P53; *Ppar*: peroxisome proliferator-activated receptor gamma gene.

For exposure groups with significant/or borderline significant effects on average gene methylation and on CpG methylation, dunn all pair test was performed to determine the effects of exposure parameters (i.e., NPs size, CNTs shape and dose). Table 1 gives the results of dunn all pair test with associated *p*-values. Compared to the vehicle group, AuNPs 60 nm exposure in mice lung tissue induced promoter hypermethylation in ataxia telangiectasia mutated (CpG#10), cyclin-dependent kinase (CpG#6) and glutathione reductase (CpG#1) genes, while promoter hypomethylation in glutathione peroxidase (CpG#3) gene (figure 3, table 3). Alterations in promoter methylation were observed for glutathione reductase (CpG#4, CpG#6) and tumor protein P53 (CpG#1) genes between low and high dose (i.e., dose effect) of AuNPs 60 nm and AuNPs 250 nm respectively (figure 3, table 1). AuNPs size effects on promoter methylation was observed for tumor protein P53 (CpG#1) gene (figure 3, table 1). CNTs exposure affected the promoter methylation of ataxia telangiectasia mutated gene (figure 4, table 1).

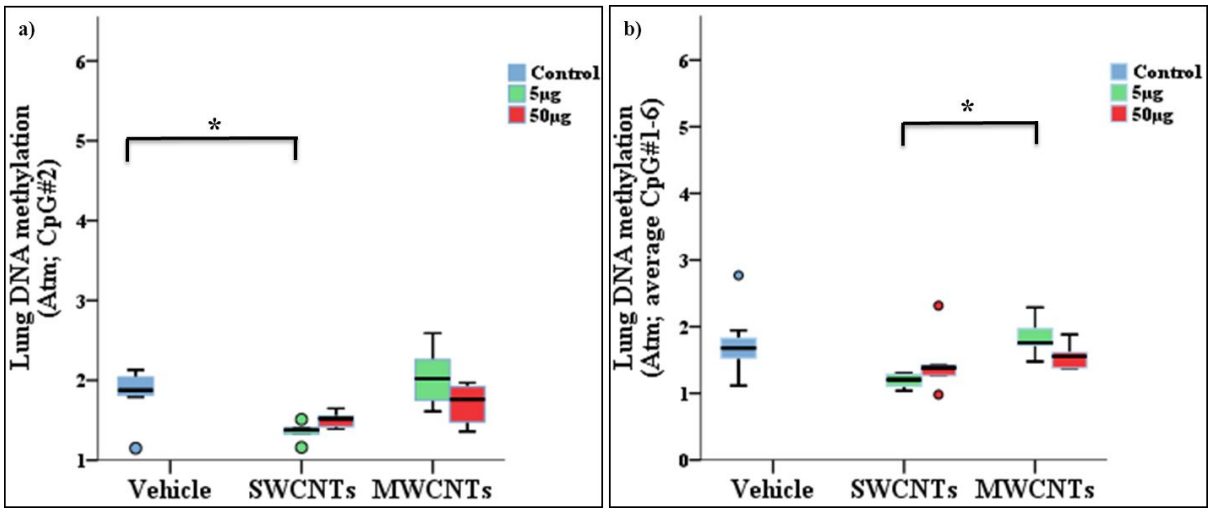

Figure 4: Effect of nanomaterial exposure on gene promoter methylation. Bars connect exposure groups with significant methylation difference. a-b): effect of single-walled and multi-walled carbon nanotubes (SWCNTs and MWCNTs) exposure on promoter methylation levels of *Atm* gene in lung tissue. In panels, box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles outside the ends of whiskers. Asterisk sign (*) shows significance levels at p=0.5. *Atm*: ataxia telangiectasia mutated.

Table 1: Effect of exposure i.e., AuNPs size, CNTs shape and dose on CpGs methylation level within promoter region(s) of gene(s). Dunn all pairs post-hoc test was used to observe factors accounted for the observed variability (*p*- value of 0.05 set to be significant).

AuNPs exposed lung DNA methylation					**CNTs exposed lung DNA methylation**				
Gene Symbol	Per Position and/or per gene	Variable 1 Mean DNA methylation (95%CI)	Variable 2 Mean DNA methylation (95%CI)	*p*-value	Gene Symbol	Per Position and/or per gene	Variable 1 Mean methylation (95%CI)	Variable 2 Mean methylation (95%CI)	*p*-value
Atm	CpG#10	Vehicle 0.912 (0.763-1.061)	AuNP 60 nm 50 µg 1.62 (1.36-1.89)	0.002	*Atm*	CpG#2	Vehicle 1.842 (1.588-2.096)	SWCNTs 5 µg 1.356 (1.197-1.514)	0.038
Cdk	CpG#6	Vehicle 0.726 (-0.113-1.565)	AuNP 60 nm 50 µg 2.638 (0.732-4.544)	0.031	*Atm*	average (CpG#1-6)	SWCNTs 5 µg 1.187 (1.046-1.329)	MWCNTs 5 µg 1.843 (1.464-2.221)	0.0316
Gpx	CpG#3	Vehicle 0.640 (0.455-0.826)	AuNP 60 nm 5 µg 0.101 (-0.071-0.273)	0.041					
Gsr	CpG#1	Vehicle 0.063 (-0.01-0.136)	AuNP 60 nm 50 µg 0.771 (-0.082-1.624)	0.034					
Gsr	CpG#4	AuNP 60 nm 5 µg 0.484 (-0.125-1.092)	AuNP 60 nm 50 µg 1.765 (1.361-2.169)	0.018					
Gsr	CpG#6	AuNP 60 nm 5 µg 0.042 (-0.074-1.158)	AuNP 60 nm 50 µg 1.11 (0.519-1.7)	0.012					
Trp53	CpG#1	AuNP 60 nm 5 µg 0.051 (-0.09-0.192)	AuNP 250 nm 5 µg 1.198 (0.458-1.937)	0.034					
Trp53	CpG#1	AuNP 250 nm 5 µg 1.198 (0.458-1.937)	AuNP 250 nm 50 µg 0.045 (-0.079-0.169)	0.028					
AuNPs exposed blood DNA methylation					**CNTs exposed blood DNA methylation**				
Pparg	CpG#3	AuNP 60 nm 5 µg 1.18 (0.502-1.858)	AuNP 60 nm 50 µg 4.813 (-0.95-10.576)	0.031					

In mice blood DNA, the only effect observed was the induction of promoter hypermethylation in peroxisome proliferator-activated receptor gamma gene (CpG#3) gene by exposure of AuNPs 60 nm high dose compared to the AuNPs 60 nm low dose (figure 3, table 1). AuNPs of 5nm did not significantly impacted CpG methylation compared to controls. Genes with significant/and or borderline significant methylation levels in response to NM exposure were involved in apoptotic process, immune system process, metabolic process and response to stimulus pathways.

5.5. Discussion

In current study, we investigated the epigenetic alterations in response to NM exposure in BALB/c mice. NM doses used to observe the epigenetic response in mice were based on the initial cytotoxicity experiments. In order to examine the cytotoxicity of NM, we examined the BAL cells. BAL cells analysis showed induction of immune response to AuNPs and CNTs exposure in BALB/c mice. NM exposure induced alterations in BAL total cell count in exposed mice. Deferential cell count revealed macrophage dominant immune response by AuNPs exposure, while CNTs exposure lead to macrophage and neutrophils driven immune response in mice. After 48 hrs of exposure, NPs were associated with pulmonary macrophages. Our findings are in agreement with previous studies, where NPs were shown to be engulfed by the pulmonary macrophages after the intra-tracheal instillation *in vivo* (Sadauskas et al., 2009). Previous studies in BALB/c mice exposed to AuNPs also demonstrated immune response dominated by macrophage; whereas SWCNTs and MWCNTs exposure in BALB/c were shown to induce macrophages as well as neutrophils influx in lung interstices (Ravichandran et al., 2011, Hussain et al., 2011). Pulmonary inflammation plays key role in various pathologies. Macrophage influx into lung interstices after AuNPs and CNTs exposure carry out cleaning function in lungs. These macrophages can take different phenotypes based environmental signals, and can enhance the oncogenic growth leading to pulmonary pathologies (Yang et al., 2012). For the selected cytokines under the current settings, we did not observe alterations in their concentrations in lung interstices after AuNPs and CNTs exposure compared to the controls. Contrary to our observation, studies have reported that exposure of certain nanomaterial to BALB/c mice is associated with changes in

cytokines concentrations in the lung tissue (Meng et al., 2011). Further, some studies also showed contradictory effects of nanomaterial exposure on inflammatory endpoints under different experiment settings (Alkilany and Murphy, 2010). These observations highlight need for the use of standardized nanomaterial applied under similar experimental settings in order to draw conclusion across studies.

To measure the induction of oxidative stress in NM exposed mice lung samples, we measured GSSG/GSH ratio. Our results showed that AuNPs and CNTs at selected doses do not induce significant alterations neither for GSSG/GSH ratio nor for mitochondrial copy member compared to the controls. Similarly, our results also did not show the induction of DNA damage in BAL cells in exposed mice compared to the controls. Comparing with the published data, our findings supports the concept that AuNPs exhibit low toxicity, as described by Chen et al (Chen et al., 2009), and that AuNPs are not genotoxic. Contrarily, some studies also have reported DNA damage and oxidative stress induction by AuNPs (Sabella et al., 2011). Use of different dose, exposure methods, *in vitro* and *in vivo* models, and even the AuNPs and CNTs purchased/manufactured from different sources/methods make it difficult to compare our findings with the published ones. Contradictory reports are published on the cellular and genotoxic effects of SWCNTs and MWCNTs (Singh et al., 2009). Some of these studies are criticized owing to high doses applied. In current study we applied low subcytoxic CNTs doses and relatively short exposure period (48 hrs), which could partly explain the contradiction on the observed effects. Previous reports have shown that the size of NPs could have strong effect on cellular activates in exposed cells (Shang et al., 2014). Chen et al., 2009 exposed BALB/c mice to AuNPs of 3nm, 5nm, 50nm, and 100 nm, and reported that AuNPs of 37nm were more harmful than other AuNPs sizes (Chen et al., 2009). Although it is difficult to compare above studies with the current one, but under the current experimental settings, we did not observe the effect of size and shape of nanomaterial on the induction of oxidative stress and DNA damage in vivo.

Important part of current study was to investigate the epigenetic alterations induced by NPs. Epigenetic alterations i.e., DNA methylation, histone modifications, microRNAs, are described in response to particulate matter (PM) and UFP (Bellavia et al., 2013). This encouraged us to investigate if NM also induce epigenetic changes

similar to their larger counterparts. To investigate the epigenetic effects, we analyzed the DNA methylation/hydroxymethylation at global and at gene promoter levels in NM exposed mice. Gene specific methylation was analyzed in mice lung and blood DNA by bisulfite-PCR pyrosequencing, while global 5mC and 5hmC was analyzed by LC-MS method only in mice lung DNA, owing to insufficient yield of DNA from mice blood. For global 5mC and 5hmC, we did not observe significant effects in exposed compared to the control mice. DNA repetitive elements constitutes ~ 50% of genome, and are highly methylated thus contributing significantly towards global DNA methylation. Also within repetitive elements, different subfamilies show differential sensitivity to environmental stressors (Byun et al., 2013). Our results indicate that BALB/c lungs are insensitive to global DNA methylation changes by AuNP of 5 nm, 60 nm and 250 nm, and SWCNTs and MWCNTs at selected doses and time. Although cells are insensitive to methylation changes globally in response to AuNPs and CNTs exposure, it is possible that the exposure leads subtle methylation changes at gene levels. Thus, we further investigated the gene specific methylation changes in exposed mice lung and blood DNA.

For gene specific methylation, we observed AuNPs size and CNTs shape, and dose specific significant alterations in gene promoter methylation in exposed mice compared to the controls. In general, we observed more genes with methylation changes in lung DNA than in the blood DNA in exposed mice compared to the controls. This is expected since lung cells were directly exposed, while the blood cells did not have direct exposure to NPs and CNTs. Interestingly, we observed more genes with promoter methylation perturbations in AuNPs exposed mice compared to the CNTs exposed mice (Table 1). This is in contrast to the current paradigm that exposure to AuNPs do not induce adverse biological response. Based on the current findings; we can report that AuNPs are potent to induce epigenetic changes compared to CNTs. It is difficult to compare current findings with previous data, especially on gene specific methylation endpoints. Currently, one study investigated the effect of nano-silicon dioxide (nano-SiO2) on *PARP-1* gene methylation *in vitro*, and reported hypermethylation of *PARP-1* gene (Gong et al., 2012). No other studies investigated the effects of NM exposure on gene methylation. We are first to report that genes are sensitive to methylation changes by the nature, size, shape and dose of NM applied *in vivo*. Our statistical analysis included the assessment of DNA methylation changes at

each CpG level, within the selected region of gene promoter sites, in response to NM exposure. Our results indicated the induction of CpG specific methylation changes in NM exposed mice. This highlighted the differential sensitivity of individual CpGs within gene promoter to NM exposure. Some previous reports described alterations in histone posttranscriptional modifications and microRNA in response to NM exposure *in vitro* (Gong et al., 2010, Li et al., 2011). Cells epigenetic marks i.e., DNA methylation, histone posttranscriptional modifications and microRNAs are described to work in concert. In line with this, current findings complement previous reports (on NM induced alterations in histone posttranscriptional modifications and in microRNAs) that NM exposure leads to epigenetics changes.

Genes with significant/and or borderline significant methylation levels in response to NM exposure were mapped to their biological processes (Table 4). Epigenetic induction of apoptotic, immune, metabolic, and response to stimulus processes, in NM exposed mice highlighted the importance of these cellular processes in NM induced stress response.

In current study, we included 17 genes for the promoter methylation analysis. These genes were selected because they are commonly affected by xenobiotic exposure. It is possible that NM exposure also induce promoter methylation changes in other genes as well, which further needs to be explored. Investigations remain in order to understand the functional importance of these epigenetic changes in nanotoxicology. Recently, cellular epigenetic stress rather than genetic events are suggested to be culprit in driving disease process. Our findings also corroborate this fact; since we observed significant gene promoter methylation alterations in NM exposed mice lung and blood DNA without significant genotoxic effects. However, the extent and duration of epigenetic stress leading to diseases needs to be fully understood.

Conclusion

We conclude AuNPs and CNTs exposure induce size, shape and dose specific DNA methylation changes *in vivo*. Although methylation changes were not observed at global scale, but gene specific methylation changes were observed in response to

AuNPs and CNTs potentially leading to adverse health consequences, and warrants further investigations. Also, our data highlights the bronchial inflammation in response to NPs and CNTs exposure. Reverse causality between observed DNA methylation changes in lung DNA and bronchial inflammation needs further investigations.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary table T1: gene specific methylation assay sequences for rodent bisulfite-PCR pyrosequencing.

Gene symbol	Forward primer	Reverse primer (biotinylated)	Sequencing primer	Annealing temperature (°C)
Atm	GGGTGTTTTAAAGGAAGAAGT	TATAACCAAAAAAAACCTAATAACC	TTTAAAGGAAGAAGT	52
Cdk	TTGTTTTTGGTTTTGTATATTATTT	TTATCAAAAACTAAACTCTCCTTAC	AAGTTTTTTATGGAG	52
Dnmt1	GTTGGTATTTTGTAGGTTGTAGA	ACATAATCTTCCCCCACTCTCTTA	TAGAATAGTTTTGAA	58.3
Gadd45a	TTTAGTAGATTTTGGGTTGTAGTTT	TAACTCTACAAATCCATTTCACCCT	TATATAGTGTAGGTT	53.9
Gpx1	TTAAAAGGAGGTGTAGGGTTTTGT	CAAAAAACCCAAACTCACAAACT	AGTATGTGTGTTGTT	58.3
Gsr	GGAGAGTTATAAGTTGGGTGGTATTT	TTTTTAACTTCAACATTACACCTATACAAA	GTTGGGTGGTATTTG	53.9
Gss	TTTTTTTTAAGGAAATTTGATTTT	ATCACTACTCATATAATACCCCTTCC	AGGAAATTTGATTTT	50
Myc	TTAAGAAGGTAGTTTTGGAGTGAGAG	AACAAAAAACACTATCCCCAAATAA	GTAAGAGTTTTTTT	53.9
Nfkb2	GTAGAATTGGAGTTGGGTGATATTT	TTACTCCTCTCCAACCAAAAAACT	AGTTGGGTGATATTT	59.4
Oxsr1	GGGAATTTGATTTTTAGTTTTTAT	AAACCTTTTATCCTAATTAACCTTC	AAAATTTTTTAGGAT	50.7
Trp53	GAATTTAAAGTAATTATTAGGGAA	AAAACCCAAAATTCAAACTACAACT	GTGTTTAAAGTTAAG	50.7
Trp73	TTGTAATTTAGGGGTTTAGGAGTGTT	AACTATAATCTCTACCAAACAAATC	GAATATTGAAAGTTT	53.9
Pparg	GGAGTTTGTGAGATTAATAGTTTGA	ATCACCTAACCAATCAAATCCAA	AGATTAATAGTTTGA	53.9
Tet1	ATTAATTTTTGGATAAATTTTTTAG	AATCATATACCTCTACCTACCTCTTCTAC	GGATAAATTTTTTAG	52
Tet2	GTTTATTTTTTGTTTATTTTGGTTA	CATTAAAAACTACTAACTTAATTCTTTC	TGTTTATTTTGGTTA	50.7
Tnf-a	TTTTTTTGGTGGAGAAAATTATGAT	CTAATTAACCCCAAATTACCACAAA	TTTATATTTTTGTTT	53.9
Xrcc1	GGTTTAATGATTAGGGTAAATTATA	AATTCCCTTAACAACAAACATTCC	AGGTTTTTAGGAAGT	52
Nfkb1	AGGGGTTTGGGTATATTTTTTTAAA	AAAAAACCCCAAACAAAAATC	GTAAGAGTTTTTTT*	53.9
Tdg	GGTTTTAAGTTTTTTTGAAGGTTT	ATTAACCTAACCAACATCAC	TTTTTTTGAAGGTTT*	52.1

* Sequencing primers did not pass the validation step for these assays.

Supplementary table T2: sequences for pyrosequencing control run.

Assay name	Assay sequence
PSQ-C	TTGCGATACGACGGGAACAAACGTTGAATTC
PSQ-T	TTGCGATACAACGGGAACAAACGTTGAATTC
Sequencing primer	AACGTTTGTTCCCGT

Supplementary table T3: Physicochemical characteristic of Au NPs used in this study.

Nanoparticle	Average diameter (nm) [TEM]*	H_2O [DLS]**		2% serum [DLS]**	
		Average hydrodynamic diameter (nm)	Zeta potential	Average hydrodynamic diameter (nm)	Zeta potential
Au NP 5 nm	5	9.6	-3.99	121.6	-18.42
Au NP 60 nm	60	69.3	-11.49	98.1	-0.54
Au NP 250 nm	250	226.5	-21.17	225.8	-7.15

*TEM: transmission electron microscopy, ** DLS: dynamic light scattering, Au NP: gold nanoparticle

Supplementary table T4: Size distribution of CNTs used in this study.

Nanoparticle	Average diameter (nm)	Average length (µm)
SWCNT*	0.8	10
MWCNT**	9.5	105

*SWCNT: single-walled carbon nanotubes, **MWCNT: multi-walled carbon nanotubes

Supplementary table T5: Effect on gene promoter methylation changes in mice lung DNA induced by exposure to gold nanoparticles (AuNPs) (table T5 a) and CNTs (table T5 b). *P*-values of Wilcoxon test statistics conducted on average methylation per gene and methylation per CpG within each gene are reported. Number of CpGs analysed are variable e.g., 10 CpGs were analysed in the promoter region of *Atm* gene while 6 CpGs were analysed in the promoter region of *Cdk* gene. Cells highlighted in red indicated the significant effects of exposure on gene promoter methylation, whereas cells highlighted in orange indicate the effect of exposure close to pre-set cut-off value of significance (borderline significant effect) (Wilcoxon test, *p*-value of 0.05 set to be significant).

Table T5-a

Gene name	% promoter methylation per CpG analyzed										Average methylation
	CpG# 1	CpG# 2	CpG# 3	CpG# 4	CpG# 5	CpG# 6	CpG# 7	CpG# 8	CpG# 9	CpG#10	Average
Atm	0.3257	0.8046	0.8746	0.2089	0.803	0.6207	0.5829	0.848	0.7524	**0.0047**	0.3813
Cdk	0.9899	0.2353	0.993	0.4541	0.2772	**0.0128**					0.1244
Dnmt1	0.0541	0.9241	0.6743	0.0757	0.4352	0.5077					0.086
Gad45 a	0.623	0.1968	0.4785	0.1458	0.4374						0.7185
Gpx	0.5123	0.6818	**0.0524**	0.5604	0.0906	0.6996	0.2329				0.2795
Gsr	**0.0074**	0.5716	0.2056	**0.0209**	0.6202	**0.0046**	0.2957				**0.0247**
Gss	0.491	0.8148	0.8558	0.3835	0.0543	0.0975					0.1156
Myc	0.9589	**0.0382**	0.4463	0.594	0.8079						0.6047
Nfkb2	0.9589	**0.0382**	0.4463	0.594	0.8079						0.6047
Oxsr1	0.439	0.3600	0.1164	0.7459	0.6121	0.5029					0.671
Trp53	**0.0054**	0.4646	0.9752	0.316	0.7373						0.195
Trp73	0.5096	0.4696	0.8897	0.4248	0.4973	0.0687	0.4644				0.4962
Pparg	0.1857	0.2319	0.3317								0.1494
Tet1	0.1432	0.1899	0.6474	0.2344							0.4266
Tet2	0.0515	0.2236	0.4958	0.2144							0.1474
Tnf-a	0.9205	0.5134	0.563								0.9662
Xrcc1	0.2505	0.6725	**0.0201**	**0.0463**							**0.0428**

Table T5-b

Gene name	% promoter methylation per CpG analyzed										Average methylation
	CpG#1	CpG#2	CpG#3	CpG#4	CpG#5	CpG#6	CpG#7	CpG#8	CpG#9	CpG#10	
Atm	0.4138	**0.0386**	0.2837	0.2381	0.0591	0.1695	0.1116	0.0787	0.1521	0.2134	**0.0341**
Cdk	0.4791	0.2092	0.9904	1.0000	0.9205	0.27073					0.8049
Dnmt1	0.4913	0.4413	0.6225	0.1473	0.2981	0.554					0.123
Gad45a	0.9297	0.1789	**0.0233**	0.0677	0.076						0.0208
Gpx	0.9445	0.4656	0.2049	0.2537	0.6287	0.3983	0.1002				0.7854
Gsr	0.8397	0.8851	0.2114	0.8429	0.9521	0.4091	0.6959				0.5762
Gss	0.3545	0.0588	0.7796	0.5792	0.5271	0.9308					0.6912
Myc	0.8819	0.6977	0.5416	0.3552	0.3511						0.5943
Nfkb2	0.8819	0.6977	0.5416	0.3552	0.3511						0.5943
Oxsr1	0.4995	0.3883	0.8502	0.6006	0.4781	0.3776					0.4406
Trp53	0.4249	0.6457	0.3662	0.3851	0.177						0.506
Trp73	0.9571	0.5316	0.7191	0.7224	0.8648	0.5531	0.4787				0.9518
Pparg	0.5558	0.1755	0.8536								0.5589
Tet1	0.1935	0.9631	0.283	0.87							0.7665
Tet2	0.8484	0.1528	0.7587	0.5324							0.3193
Tnf-a	0.7754	0.6052	0.4227								0.7282
Xrcc1	0.8234	0.6512	0.4549	0.4296							0.664

Supplementary table T6: Effect on gene promoter methylation changes in mice blood DNA induced by exposure to gold nanoparticles (AuNPs) (table T6 a) and single and multi-walled carbon nanotubes (table T6 b). *P*-values of Wilcoxon test statistics conducted on average methylation per gene and methylation per CpG within each gene are reported. Number of CpGs analysed are variable e.g., 8 CpGs were analysed in the promoter region of *Atm* gene while 4 CpGs were analysed in the promoter region of *Cdk* gene. Cells highlighted in red indicate the significant effects of exposure on gene promoter methylation, whereas cells highlighted in orange indicate the effect of exposure close to pre-set cut-off value of significance (borderline significant effect) (Wilcoxon test, *p*-value of 0.05 set to be significant).
DNA coordinates of CpGs analysed in each bisulfite-PCR pyrosequencing promoter assay were same between nanomaterial (AuNPs and CNTs) exposed lung and blood DNA samples (table T5 a-b and table T6 a-b). However, CpGs in blood DNA (table T6 a-b) that did not pass the quality control were discarded from the analysis (e.g., *Atm*: CpG#9, CpG#10 were discarded).

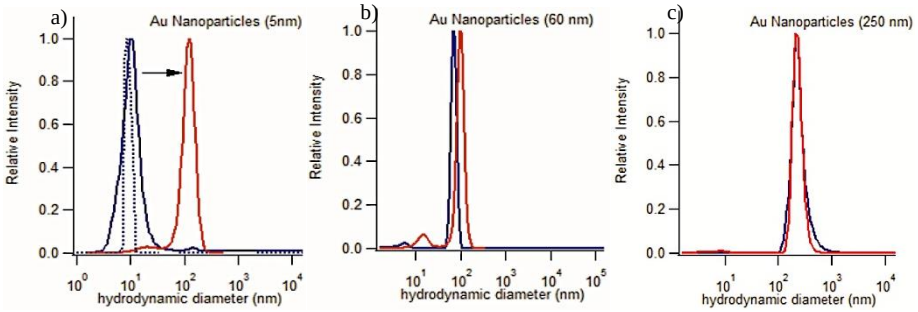
Table T6-a

Gene name	**% promoter methylation per CpG analyzed**								**Average methylation**
	CpG#1	CpG#2	CpG#3	CpG#4	CpG#5	CpG#6	CpG#7	CpG#8	Average
Atm	0.5516	0.1097	0.2099	0.1476	0.4140	0.9800	0.2440	0.0518	0.0937
Cdk	0.6546	0.8591	0.2216	0.7286					0.9245
Dnmt1	0.6771	0.7706	0.4168	0.4367					0.0492
Gad45a	0.5658	0.7372	0.1964	0.8110	0.2403	0.4333			0.1250
Gpx	0.7089	0.3167	0.1554	0.4168					0.8460
Gsr	0.0289	0.3296	0.9747	0.5951	0.6961				0.7558
Gss	0.1712	0.6327	0.8110	0.8298	0.1849				0.5645
Myc	0.5179	0.6050	0.0725	0.2900	0.0648				0.2296
Nfkb2	0.7646	0.8653	0.7866	0.0284					0.4722
Oxsr1	0.6496	0.0421	0.2984	0.0836	0.2387	0.2637	0.2219		0.1410
Trp53	0.4593	0.5987	0.5980	0.1730	0.9058				0.9147
Trp73	0.9528	0.4376	0.3356	0.6810	0.6781	0.2334			0.4677
Pparg	0.2731	0.4694	0.0266						0.1774
Tet1	0.1200	0.6402	0.2331						0.6766
Tet2	0.6085	0.3630	0.7674	0.8278	0.0632	0.1547			0.4022
Tnf-a	0.2409	0.5941	0.0618						0.3339
Xrcc1	0.1310	0.7489	0.5490	0.4880					0.2913

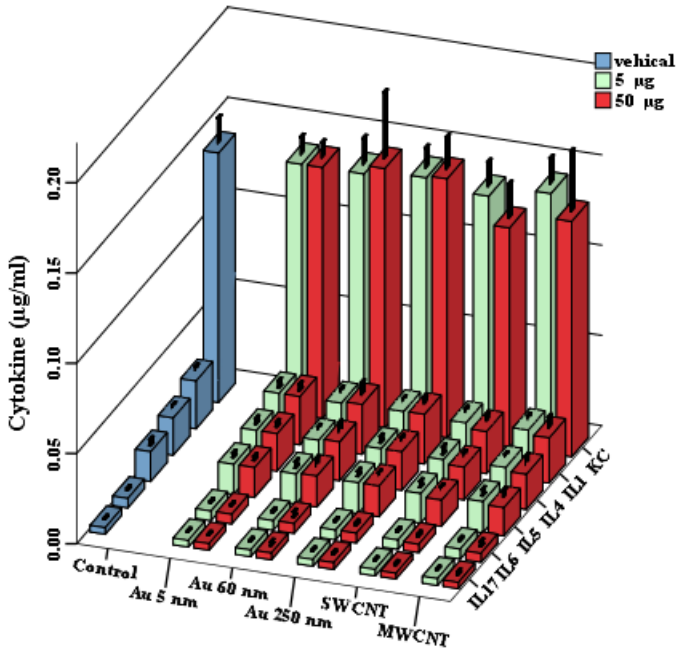
Table T6-b

Gene name	% promoter methylation per CpG analyzed								Average methylation
	CpG#1	CpG#2	CpG#3	CpG#4	CpG#5	CpG#6	CpG#7	CpG#8	Average
Atm	0.5197	0.3225	0.7894	0.3572	0.2222	0.0735	0.3668	0.3698	0.3438
Cdk	0.5753	0.8984	0.4204	0.0843					0.4719
Dnmt1	0.3472	0.4760	0.3502	0.8024					0.3053
Gad45a	0.4004	0.6074	0.6911	0.3233	0.8528	0.9192			0.6850
Gpx	0.0314	0.2887	0.2532	0.3526					0.5776
Gsr	0.6608	0.8585	0.8389	0.7721	0.2066				0.9513
Gss	0.4128	0.5522	0.7695	0.9210	0.1153				0.5591
Myc	0.2901	0.5256	0.5641	0.7266	0.7149				0.4488
Nfkb2	0.1004	0.7606	0.7383	0.5800					0.7004
Oxsr1	0.1943	0.2633	0.5936	0.3783	0.7116	0.1427	0.1254		0.1136
Trp53	0.8158	0.2784	0.7899	0.3486	0.7920				0.3903
Trp73	0.8472	0.1694	0.1500	0.9733	0.7635	0.9052			0.5599
Pparg	0.3358	0.5994	0.5078						0.9370
Tet1	0.5861	0.6663	0.1443						0.9303
Tet2	0.2455	0.3804	0.9849	0.1545	0.5037	0.5114			0.4169
Tnf-a	0.5134	0.5823	0.8623						0.5642
Xrcc1	0.5945	6242.0000	6545.0000	0.6420					0.8234

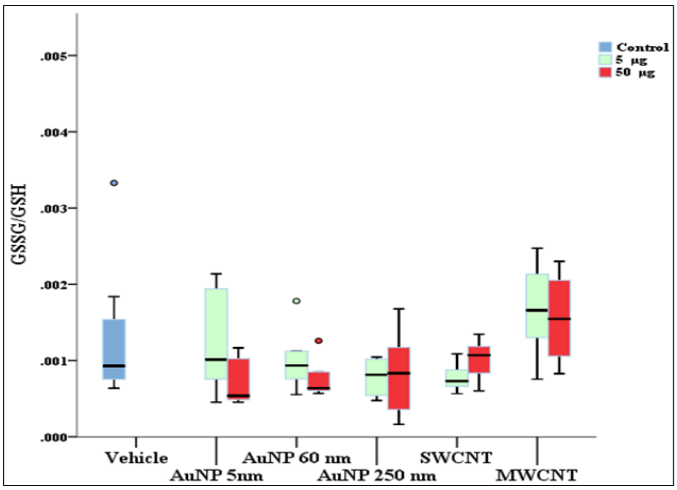
Supplementary figure F1: Dynamic light scattering (DLS) size distribution of AuNPs 5nm (a), AuNPs 60nm (b) and AuNPs 250nm (c) in H_2O (blue curve) and in 2 % serum (red curve). Au: gold.



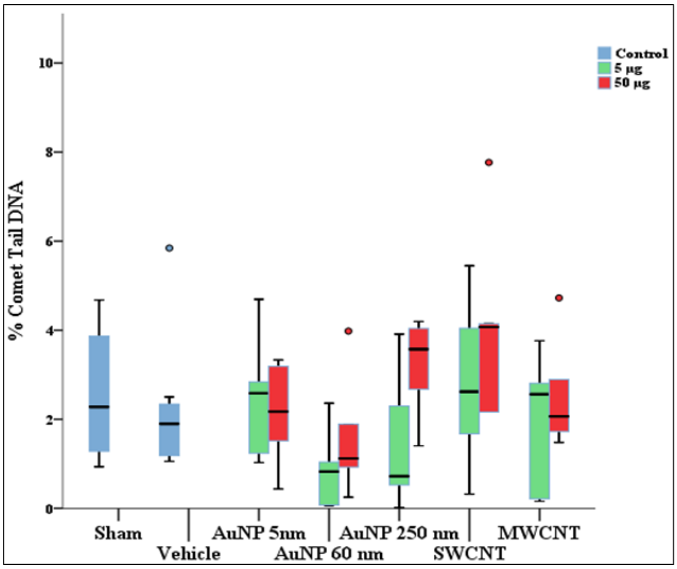
Supplementary figure F2: Cytokine levels were measured using flow cytometry. For the selected cytokines, we did not observe significant difference (KC: p-value=0.663; IL1: p-vaue=0.66; IL4: p-value=0.66; IL5: p-value=0.663; IL6: p-value=0.663; IL17: p-value=0.661) between AuNPs and CNTs exposed and control groups. Data represented as mean ±SD. Significant level was set at 0.05.



Supplementary figure F3: Oxidative stress in mice lung samples in response to AuNPs and CNTs exposure. GSSG/GSH (Wilcoxon test; p=0.173) ratio was measured in mice lung samples in order to quantify the levels of oxidative stress. GSSG: oxidized form of glutathione disulfide; GSH: reduced glutathione. In the panel, box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles (panel a) outside the ends of whiskers.



Supplementary figure F4: DNA damage profile of AuNPs and CNTs in exposed and control mice. DNA damage was assessed by comet assay. Comet tail is a marker of DNA damage, that was not significant (Wilcoxon test; p=0.486) between exposed and control samples. In figure, box plot describes the median (line across the box), inter-quartile range and maximum and minimum values (whiskers). Outliers are shown as colored circles outside the ends of whiskers.



6. Chapter 6

Adopted from:

Global methylation and hydroxymethylation in DNA from blood and saliva in healthy volunteers

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Abstract

Aims: We describe a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify and compare simultaneously global methylation and hydroxymethylation in human DNA of different tissues. ***Materials and Methods:*** Blood and saliva DNA from fourteen volunteers was processed for epigenetic endpoints using LC-MS/MS and PCR-pyrosequencing technology. ***Results:*** Global DNA methylation was significantly lower in saliva (mean 4.61%+/-0.80%), compared to blood samples (5.70%+/-0.22%). In contrast, saliva (0.036%+/-0.011%) revealed significantly higher hydroxymethylation compared to blood samples (mean 0.027%+/-0.004%). Whereas we did not find significant correlations for both epigenetic measures between the tissues, a significant association was observed between global methylation and global hydroxymethylation in saliva DNA. Neither LINE-1 nor Alu elements of blood and saliva correlated, nor were they correlated with the DNA hydroxymethylation of blood or saliva respectively. ***Conclusion:*** Global DNA methylation and hydroxymethylation of cytosine can be quantified simultaneously by LC-MS/MS. Saliva-DNA cannot be considered as a surrogate for blood-DNA to study epigenetic endpoints.

6.1. Introduction

Epigenetics refers to the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence (Wu and Morris, 2001). Epigenetic mechanisms are essential for development (genome imprinting, X chromosome inactivation, etc.) and differentiation (transcriptional regulation), but can be disrupted by exogenous agents (Bird, 2007). Epigenetic changes have been described in relation to environmental exposure similar to changes observed in chronic diseases, such as cancer or Alzheimer disease. The most investigated epigenetic mechanisms include DNA methylation, histone modifications and RNA-mediated silencing (Jaenisch and Bird, 2003, Feinberg et al., 2006).

DNA cytosine methylation (5mC) is chemically relatively stable, but still, dynamic epigenetic modifications regulated by DNA methyltransferases (DNMTs) often occur. These alterations involve the covalent addition of a methyl group to the 5-position of cytosine with S-adenosyl methionine (SAM) as the methyl donor (Webster et al., 2013, Barros and Offenbacher, 2009). DNA methylation is almost exclusively restricted to CpG dinucleotides clustered within the gene promoter and in repeated elements such as long (LINE-1) and short (Alu) interspersed elements (Webster et al., 2013, Kile et al., 2010). Paradoxically, 90% of the methylated CpG lies outside the coding regions such as CpA, CpT, and CpNpG sites, possibly to serve as repressors of transposons or viral-like transcripts (Baccarelli and Ghosh, 2012). In general, hypermethylation of the DNA promoter regions inactivates the gene expression and hypomethylation activates the expression (Klein and Costa, 1997). Aberrant 5mC levels, i.e., global hypomethylation and/or gene-specific hypermethylation or hypomethylation, are observed in diseases like leukemia and cancer, but have also been observed in cells exposed to carcinogenic agents (Baccarelli and Bollati, 2009, Godderis et al., 2012, Bollati et al., 2007).

Besides 5mC, other epigenetic modifications such as 5-hydroxymethylcytosine (5hmC), have been recently discovered in this rapidly evolving field. Significant levels of 5hmC have been found in DNA from embryonic stem cells, neurons and brain (Shock et al., 2011). Even though the biological function is not yet completely clarified, 5hmC is of special interest in order to understand the regulation of gene

expression and chromatin structure since it acts as an oxidized intermediate in the active demethylation of 5mC or even may be the final product of genome-wide demethylation (Shock et al., 2011, Wang et al., 2012).

The conversion of 5mC to 5hmC is catalysed by ten eleven translocation (TET) family of proteins (TET1, TET2 and TET3) which are 2-oxoglutarate (2OG)- and Fe(II)-dependent dioxygenases. TET1 and TET2 are involved in the maintenance of embryonic stem cells pluripotency and cell lineage commitment. TET1 is a fusion partner of the MLL gene in rare cases of acute myeloid and lymphoid leukemias. TET2 modulates the balance between self-renewal and differentiation in hematopoietic stem cells, making them critical for normal myelopoiesis. Loss-of-function of TET2 is associated with acute myeloid leukemia, myelodysplastic and myeloproliferative disorders. TET3 contributes to the global DNA demethylation during the zygotic stage of embryonic development (Wang et al., 2012). These studies highlight the role of TET-mediated 5-hmC in the developmental processes, and the possibility that altered DNA 5hmC levels can lead to malignancy (Webster et al., 2013, Wang et al., 2012).

Beside the TET pathway, 5hmC can also be formed by other mechanisms, e.g. UV irradiation of 5mC in aerated aqueous solution and DNA methyltransferase reaction of cytosine with formaldehyde. The process of active DNA demethylation via 5hmC also seems to be mediated by activation-induced deaminase and DNA glycosylase, which are involved in deaminating and excision repair (Webster et al., 2013). This indicates that environmental factors might modify the DNA methylation and hydroxymethylation status, and that assays are needed to measure both total DNA methylation and hydroxymethylation (Lewandowska and Bartoszek, 2011).

In-vivo studies on the epigenetic effects of environmental carcinogens are scarce, and have mainly been performed on human lymphocytes (Bollati et al., 2007, Godderis et al., 2012). Blood samples are often the preferred source of genetic material because they provide large amounts of cells and in the same sample a wide range of environmental agents can be determined (Hansen et al., 2007). The desire for large-scale epidemiological studies involving thousands of participants necessitates less invasive and more cost-efficient procedures for collecting DNA that would facilitate

the trial recruitment (Rogers et al., 2007). Saliva and buccal swab samples are described as a non-invasive alternative to collect human DNA for epigenetic epidemiological studies (Philibert et al., 2008, Sapienza et al., 2011). Previous research showed that sufficient amounts of high-quality DNA could be collected from saliva (Quinque et al., 2006). The potential advantages of saliva sample collection compared with blood sample collection include lower overall cost, lower infection risk, increased patient convenience, acceptability and compliance (Abraham et al., 2012). However, a potential drawback is the presence of exogenous DNA (e.g. from bacteria) commonly present in human saliva and buccal swab samples (Herraez and Stoneking, 2008).

In this paper we report on the application of a fast and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of 5mC and 5hmC in human DNA from different tissues. We investigated the association between both epigenetic marks and compared the results with LINE-1 and Alu methylation, often used as surrogates for global DNA methylation in monitoring studies, determined with PCR-pyrosequencing. In addition, we also investigated for the first time whether these epigenetic endpoints in DNA of saliva are comparable with DNA from blood of human volunteers in order to serve as a non-invasive alternative for biomonitoring purposes.

6.2. Material and methods

6.2.1. Study design and Population

Fourteen healthy volunteers (n=4 male, n=10 female) aged less than 45 years were enrolled in this study. Most of the volunteers (n=12) were Caucasian. The participants were recruited among the scientific staff of the department of Oral Health Sciences and the department of Public Health and Primary Care and among KU Leuven pregraduate medical students. All participants received information about the purpose and objectives of the study and gave written informed consent to the proposed processing of the data. Participants were asked to fill out a small questionnaire on general health and lifestyle. The study was approved by the Commission for Medical Ethics of UZ Leuven (reference number: S53445).

6.2.2. Sample Collection

Donors were refrained from eating and drinking for at least 8 hours prior to sample collection and were asked to rinse their mouth prior to sample taking. Subjects were asked to produce 2 mL unstimulated saliva using the self-collection kit OG-500 from Oragene (DNA GenoTek, Ottawa, OT, Canada). Next, blood was drawn from each participant (three EDTA tubes of 4,5 mL).

6.2.3. DNA extraction

DNA extraction was performed with GeneCatcher™ gDNA Blood Kit for blood samples and Oragene OG-500 kit (DNA GenoTek) for saliva samples. The quantity and purity of DNA was determined by a NanoDrop spectrophotometer.

6.2.4. DNA methylation and DNA hydroxymethylation analysis

DNA was analyzed by LC-MS/MS as described previously (Song et al., 2005). Briefly, isolated genomic DNA samples (1 µg) were enzymatically hydrolyzed to individual deoxyribonucleosides by a simple one-step DNA hydrolysis procedure. A digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and benzonase® Nuclease to Tris-HCl buffer. Extracted DNA was hydrolyzed by adding 50 µl digest mix and incubating at 37°C for at least 8 h. After hydrolysis, 900 µL of HPLC-grade water was added to each sample. Exposure to daylight was avoided over the entire sample preparation procedure in order to minimize potential deamination of the target compounds.

Stock solutions of 5-methyl-2'-deoxycytidine (5mdC), 5-hydroxymethyl-2'-deoxycytidine (5hmdC) and 2'-deoxycytidine (dC) were prepared by dissolving commercial solid reference standards in HPLC-grade water. Stock solutions were used to prepare calibration standards. Global DNA methylation and hydroxymethylation was obtained by quantifying 5mdC, 5hmdC and dC using ultra-pressure liquid chromatography (UPLC), in combination with tandem mass spectrometry (MS-MS). LC/MS-MS analysis of the samples was conducted on a

Waters® Acquity UPLC™, coupled to a Waters® Micromass Quattro Premier™ Mass Spectrometer using electro spray ionization (ESI). A 15 µL aliquot of the sample was introduced on an Acquity UPLC BEH C_{18}, 50 mm x 2.1 mm, 1.7 µm column, held at a temperature of 40°C. The mobile phase used for the chromatographic separation was a mixture of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) using the following gradient: the program was starting at 10%B, increasing linearly to 100%B for 2 min, then hold from 2 to 2.1 min at 100%B and finally brought back to the initial status from 2.1 to 3.0 min. A flow rate of 0,35 mL/min was applied. The analyses were performed in the positive ESI mode and a multiple reaction monitoring (MRM) method was used with argon as the collision gas.

All DNA samples isolated from blood and saliva were hydrolysed in triplicate. Each sample was then analysed twice using the LC-MS/MS method. By interpolation from the established calibration curves, the absolute concentrations, expressed in ng/mL, for 5mdC, dC and 5hmdC, present in the samples (1 µg DNA / mL), could be derived. Together with every set of 14 volunteer DNA samples, also 3 quality control (QC) DNA samples have been prepared and analysed, in order to uncover any potential errors upon sample preparation and analysis. Global DNA methylation is expressed as a percentage of 5mdC versus the sum of 5mdC, 5hmdC and dC [%Methylation = 5mdC / (5mdC + 5hmdC+ dC)], while global DNA hydroxymethylation is expressed as a percentage of 5hmdC versus the sum of 5mdC, 5hmdC and dC [%Hydroxymethylation = 5hmdC / (5mdC + 5hmdC + dC)].

6.2.5. Pyrosequencing of LINE-1 and Alu elements

Long interspersed nucleotide elements (LINE-1) and the AluSX (Alu) methylation levels were assessed using PCR-pyrosequencing of bisulfite-treated DNA. Details of the PCR-pyrosequencing assays used in the current study are described by Kile. M, 2010 (Kile et al., 2010).

6.3. Statistical analysis

Differences between groups were analysed by Mann Whitney *U* test for unrelated data and Wilcoxon signed rank test for related data. Within tissue and between tissue

DNA association of methylation and hydroxymethylation levels was investigated by spearman correlation.

6.4. Results

6.4.1. Characteristics of participants and method

A total of 14 healthy subjects (4 male, 10 females) enrolled in the study. Mean age was 29 years (range 22-43) and BMI ranged between 20 and 29 (mean 24). 6 subjects reported a history of allergy of dust mites, hay fever, asthma, eczema, penicillin or nickel allergy.

The OrageneTM saliva collection kit yielded sufficient DNA out of 2 mL saliva for all participants with a mean yield of 20.1 µg DNA (range 6.8-135.4 µg DNA). To perform LC-MS analysis, a minimum concentration of 20 ng DNA/µL is required. We extracted on average 42.4 µg DNA (range 1.8-97.4 µg) out of 4.5 mL blood. The minimum 260/280 ratio was > 1.6 for DNA of both tissues.

In order to perform the DNA (hydroxy)methylation analysis of the samples, a calibration series in HPLC-grade water was prepared for 5mdC, dC and 5hmdC and run by the analytical method. Calibration standards were prepared, starting from purchased reference standards, in a range of respectively 0.01 – 5 ng/mL for 5mdC, 0.2 – 50 ng/mL for dC and 0.005 – 0.07 ng/mL for 5hmdC. The same calibration standards were used in all experiments. Upon LC/MS-MS analysis, independent quantification of 5mdC, dC and 5hmdC was possible since different, unique transitions from precursor ion to product ion were monitored in MRM mode : m/z 242.2 → 125.95 for 5mdC (cone voltage 20 V, collision energy 14 eV), m/z 228.1 → 111.9 for dC (cone voltage 18 V, collision energy 12 eV) and m/z 258.0 → 141.9 for 5hmdC (cone voltage 15 V, collision energy 10 eV) . The dwell time per transition was 90 ms. Calibrations solutions were analysed in MRM mode and data processing was based on absolute peak areas of the different unique product ions. In Figure 1, the UPLC chromatograms are shown for dC, 5mdC and 5hmdC, for a volunteer's blood

and saliva sample, as well as for a calibration standard. For the three different compounds, calibration curves were constructed, as presented in Figure 2.

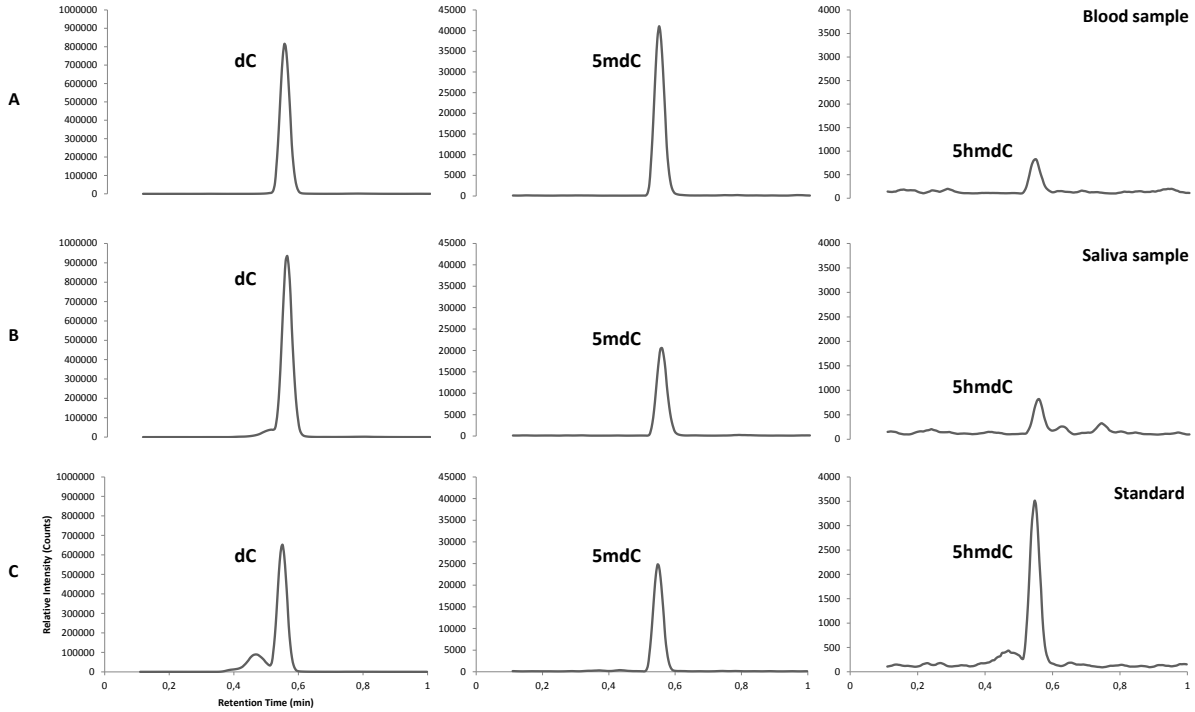

Figure 1: UPLC-chromatograms of the monitored ion transitions for 2'-deoxycytidine (dC), 5-methyl-2'-deoxycytidine (5mdC), and 5-hydroxymethyl-2´-deoxycytidine (5hmdC):
A) blood sample from a volunteer, B) saliva sample from the same volunteer and C) calibration standard, containing dC (30.7 ng/mL), 5mdC (1.5 ng/mL) and 5hmdC (0.07 ng/mL).

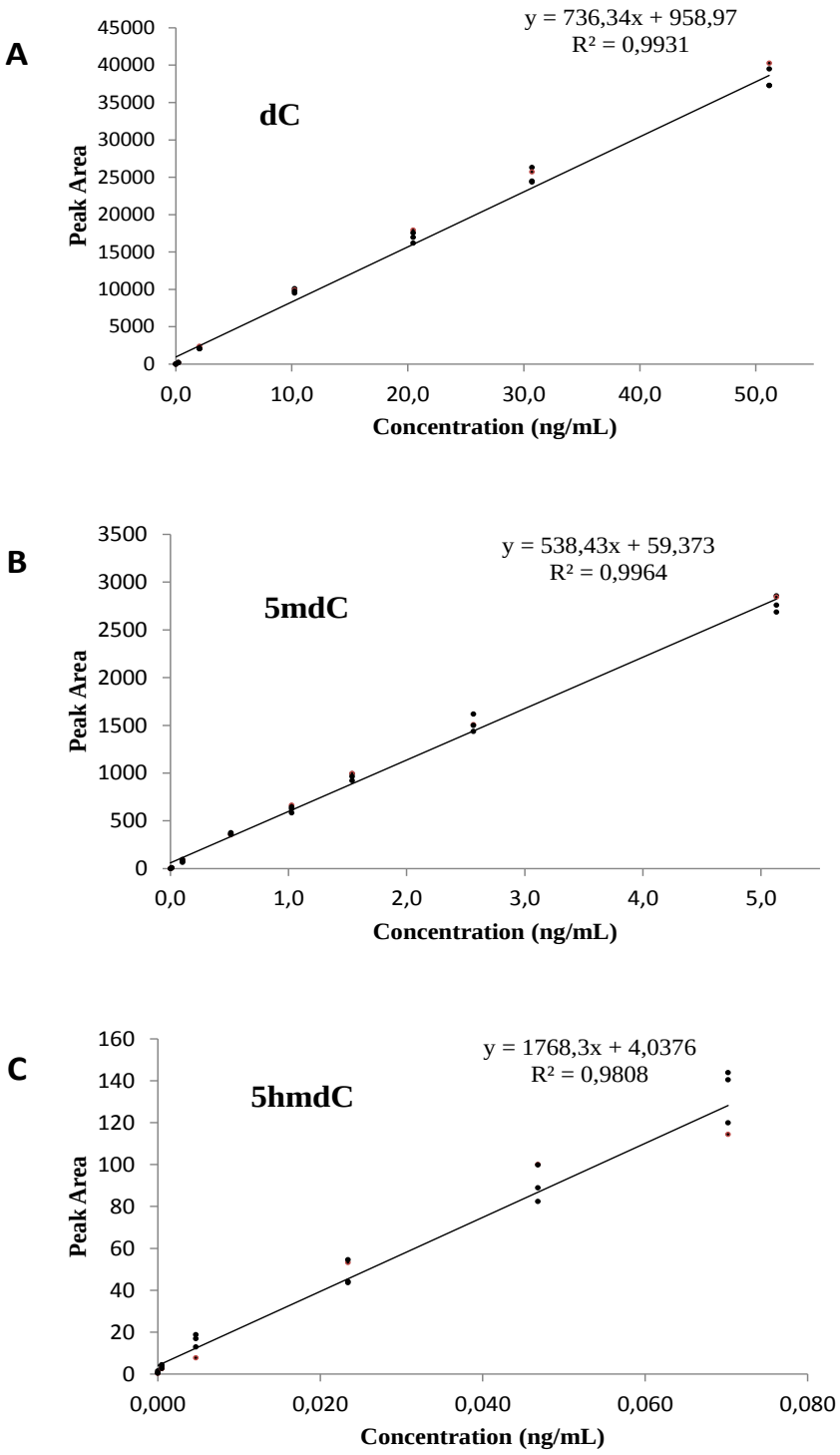

Figure 2: Calibration curves for A) 2'-deoxycytidine (dC), B) 5-methyl-2'-deoxycytidine (5mdC), and C) 5-hydroxymethyl-2´-deoxycytidine (5hmdC).

For 5mdC and dC the established linear calibration curves had correlation coefficients > 0.99 while for 5hmdC a correlation coefficient around 0.98 was observed (see Figure 2). It should be noted however that the concentration range for 5hmdC is situated far below the ones for 5mdC and dC. Using an amount of 1 µg digested

DNA, the method's limits of detection (LODs) for 5 mdC, dC and 5hmdC were respectively 0.01 ng/mL, 0.01 ng/mL and 0.005 ng/mL. As a sample aliquot of 15 µL is injected, these LODs correspond to amounts of 0.62 fmol 5mdC, 0.66 fmol dC and 0.29 fmol 5hmdC injected on-column. These LODs are comparable to previously LC/MS-MS sensitivity data, reported by Thuc et al. (2011) (0.5 fmol for 5mdC and 5hmdC) (Thuc et al., 2011).

6.4.2. Results of %methylation and %hydroxymethylation

In table 1 the results of % DNA methylation and % DNA hydroxymethylation are presented. The mean value of global DNA methylation was significantly (p=0.001) lower in saliva samples (mean 4.61%), compared to blood samples (mean 5.70%). This is in contrast with global hydroxymethylation, which was significantly higher (p=0.001) in saliva samples (mean 0.036%), compared to blood samples (mean 0.027%).

We did not observe significant differences in global DNA methylation and hydroxymethylation levels between males and females. In contrast, 6 individuals with allergy (0.029% +/- 0.002%, 0.027% - 0.032%) showed a small but significant increase (p=0.042) in %hydroxymethylation in DNA from blood compared to 8 non-allergic participants (0.025% +/- 0.004%; 0.021% - 0.031%). Neither age, nor BMI seemed to influence the epigenetic endpoints, however only subjects under 45 were allowed to participate.

Table 1: %methylation and %hydroxymethylation in blood and saliva

	Mean	Standard deviation	Minimum	Maximum
Saliva DNA % hydroxymethylation	0.036	0.011	0.019	0.053
Blood DNA % hydroxymethylation	0.027	0.004	0.021	0.032
Saliva DNA % methylation	4.61	0.80	2.36	5.75
Blood DNA % methylation	5.70	0.22	5.25	6.12

Next, we compared %DNA methylation in blood and saliva; and %DNA hydroxymethylation in blood and saliva. No significant association could be observed

(spearman rho=0.141, p=0.631 and -0.021, p=0.943) for both epigenetic endpoints between the two tissues.

We also performed the correlation between %DNA methylation and % DNA hydroxymethylation in the same tissue. A significant association was found between % methylation and %hydroxymethylation in saliva DNA (spearman rho=0.716; p=0.004) (Figure 3A). In blood, no such significant correlations could be revealed (spearman rho=0.056; p=0.850) (Figure 3B).

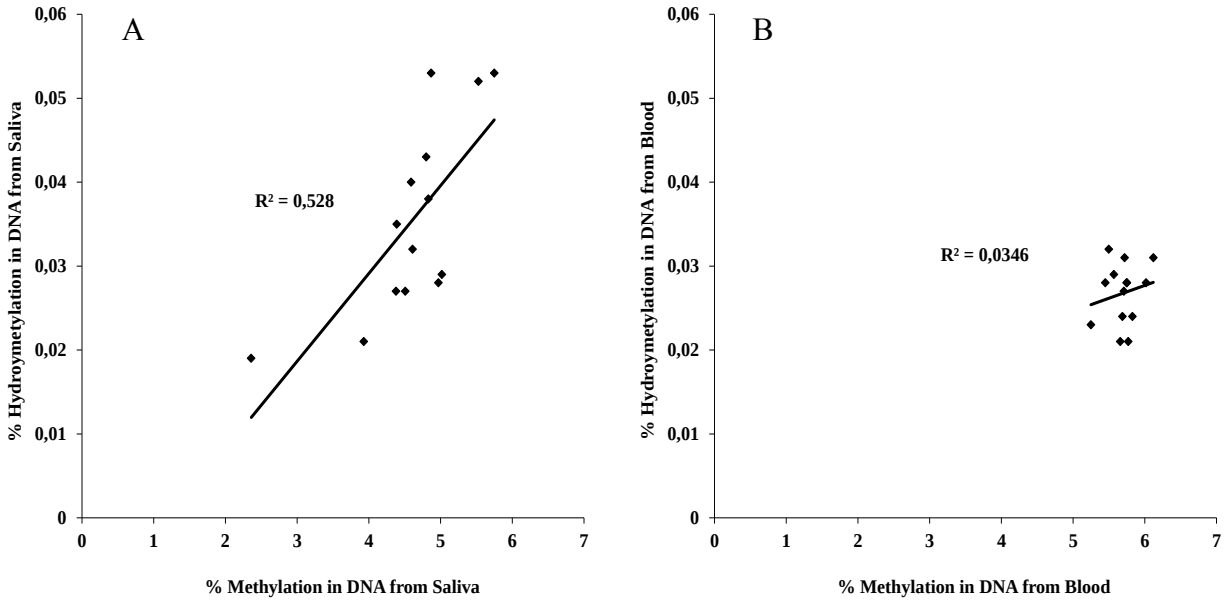

Figure 3: Correlation between %DNA methylation and % DNA hydroxymethylation in the same tissue

6.4.3. LINE1 and Alu methylation

It is suggested that analyzing the methylation of DNA repetitive elements can serve as a surrogate marker for global genomic DNA methylation. We thus assessed the methylation levels of LINE-1 and Alu elements of blood and saliva DNA (supplementary table1). No significant correlation was observed between the methylation levels of blood and saliva Alu elements (spearman rho=0.297, p=0.303). Methylation levels of blood and saliva LINE1 elements were also not significantly correlated (spearman rho=-0.196, p=0.503). Although in general lower methylation levels were observed for LINE1 and Alu elements in saliva DNA compared to their

methylation levels in blood DNA (supplementary table 1). Also, no significant correlations were observed in the methylation levels of Alu (spearman rho=-0.117, p=0.690) and LINE elements (spearman rho=0.155, p=0.598) of saliva with the DNA hydroxymethylation in saliva, and in the methylation levels of Alu (spearman rho=0.139, p= 0.635) and LINE1 elements (spearman rho=0.099, p=0.737) of blood with the DNA hydroxymethylation level of blood.

6.5. Discussion

We described the method for simultaneous quantification of global DNA methylation and hydroxymethylation by LC-MS/MS with a high sensitivity and accuracy. The DNA methylation pattern is in line with a previously published study (Thompson et al., 2013). By applying the calibrated assay we showed that 5hmC is present in DNA of different human tissues. Little is known about the global 5hmC levels in different species. We report lower global 5hmC contents compared to the other studies reporting global DNA 5hmC contents in different non-human tissues. Different detection and expression methods for 5hmC used in small number of studies investigating the global DNA 5hmC contents make it difficult to compare them with our findings of global DNA 5hmC levels in blood and saliva.

Global DNA methylation levels in males and females were comparable, which is in line with published data (Weber et al., 2005). Gender also does not seem to affect global hydroxymethylation levels in DNA from blood or saliva. Since we limited the age at participation, we cannot draw conclusion on the absence of an age-effect on global DNA hydroxymethylation. The effect of age on global DNA methylation has been widely discussed (Zhu et al., 2012). Several studies based on relatively small study samples (between 76 and 237 subjects) reported an inverse association between age and genomic 5mC content from blood of healthy subjects (Zhu et al., 2012). In contrast, other studies of similar size or larger (between 32 and 526 samples) reported no association of age with genomic 5mC content (Moore et al., 2008).

One of the objectives was to determine whether saliva could be a reliable source of DNA and serve as a non-invasive alternative of blood DNA in biomonitoring studies.

Sufficient good quality DNA could be extracted from saliva and both quality and quantity were in line with previously published data (Hansen et al., 2007, Rylander-Rudqvist et al., 2006, Rogers et al., 2007). We did not observe any association between the two tissues for both epigenetic endpoints (global DNA methylation and DNA hydroxymethylation), and also no correlation was observed between the LINE1 and Alu elements of both tissues. On one hand, this could be due to the lack of power due to the small sample size or due to differences DNA extraction methods (Rylander-Rudqvist et al., 2006).

On the other hand, it is known that saliva samples are contaminated with DNA from oral bacteria and/or food, which can overestimate the amount of DNA in these samples (Hansen et al., 2007, Rogers et al., 2007, Abraham et al., 2012). In our study, participants were refrained from eating and drinking 8 hours prior to the sample collection and mouth was rinsed prior to sampling. In addition, the Oragene sample kit contains an antibacterial agent, which also prevents the growth of bacteria between the time of collection and the time of DNA purification. Immediately after collection, the samples were stored at -80°C to avoid bacterial growth. Previous studies have shown that buccal swabs contain around 11% human DNA, whereas saliva samples yield on average 68% human DNA (Garcia-Closas et al., 2001, Rylander-Rudqvist et al., 2006).

Both blood and saliva contain a variety of cell types, with different function and half-life, and presumably different susceptibility to external factors. DNA extracted from blood samples typically originates from leucocytes (granulocytes, lymphocytes and monocytes), whereas human DNA from buccal swabs mainly stems from exfoliated epithelial cells. Human DNA from saliva on the other hand is derived both from leucocytes (granulocytes, lymphocytes and monocytes) and exfoliated epithelial cells (Vidovic et al., 2012). Blood, saliva and buccal swabs not only differ in types of the cells they contain, but also in the viability of the cells. DNA from blood mostly stems from viable cells, whereas many cells in buccal swabs are dead (exfoliated cells). These differences might explain the differences in methylation pattern between the different tissues.

Our results indicated positive association for global DNA methylation and hydroxymethylation within the same tissue; i.e. saliva. Interestingly, the direction of difference between global DNA methylation and hydroxymethylation levels in saliva and blood is opposite to each other; i.e. low global DNA methylation and high global DNA hydroxymethylation in saliva compared to blood. This supports the hypothesis that 5hmC is involved as an intermediate in the active demethylation of 5mC. No association in methylation levels of blood and saliva LINE1 and Alu elements was observed with the DNA hydroxymethylation levels in blood and saliva DNA respectively. Since LINE1 and Alu elements methylation does not represent the complete cellular pool of global DNA methylation, this might explain the lack of this association along with other factors e.g., small sample size. DNA methylation and hydroxymethylation are tightly regulated in live cells. Saliva comprises mainly dead cells, which can explain the low levels of global DNA methylation but high levels of global DNA hydroxymethylation (i.e., dead cells lose control in methylating the repetitive elements which would increase the global DNA hydroxymethylation) as compared to the blood, which contains the live cells tightly regulating their genome. The mechanisms explaining how this process is controlled and mediated in different cell types is still unclear (Severin et al., 2013).

Conclusion

In conclusion: both global DNA methylation and hydroxymethylation of cytosine can be quantified simultaneously by LC/MS-MS. Global DNA methylation and hydroxymethylation in saliva and blood DNA do not seem to be comparable and consequently saliva cannot be considered as a surrogate for blood for epigenetic endpoints. There are indications of a positive association between global DNA methylation and hydroxymethylation within the same tissue; i.e. saliva.

Future perspectives

Epigenomics is an active research field driven by the massive amount of new information. New cellular pathways are emerging as knowledge of epigenomics is growing. Recently, the identification and tissue-specific distribution of DNA hydroxymethylation lead to speculation that DNA hydroxymethylation is not just a

passive mark but could play an important cellular function. Further research in tissue-specific correlation of different epigenetic factors will help understanding how epigenetic factors play a role in regulating the activity of genes.

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Financial & competing interests disclosure

Nothing to declare

Executive Summary

Introduction

- Saliva samples are described as a non-invasive option to collect human DNA for epigenetic epidemiological studies.
- We describe a sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of global DNA methylation and global DNA hydroxymethylation at cytosine in blood and saliva.

Population & Methods

- Blood and saliva DNA from fourteen healthy volunteers was processed for epigenetic measures.

Results

- Global DNA methylation at cytosine was significantly lower in saliva samples compared to blood samples.
- Saliva samples revealed higher global hydroxymethylation compared to blood samples.
- Saliva DNA showed significant association between global methylation and global hydroxymethylation.

Discussion & Conclusion

- Sensitive quantification of global DNA methylation and global DNA hydroxymethylation can be performed from the same samples using LC-MS/MS method.
- Saliva DNA cannot be considered as a surrogate for blood-DNA to study epigenetic endpoints.

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7. Chapter 7

Adapted from:

Assessment of repetitive elements DNA methylation by pyrosequencing

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Abstract

Transposable elements (TE) comprise half of the human genome. LINE-1 and *Alu* are the most common TE, and they have been used to assess changes in the DNA methylation of repetitive elements in response to intrinsic and extrinsic cellular events. Pyrosequencing is a real-time sequencing technology that enables quantitative assessment of TE methylation at single-base resolution. In pyrosequencing, a region of interest is first amplified from bisulfite-converted DNA by polymerase chain reaction (PCR), before PCR amplicons are rendered single stranded and annealed with the pyrosequencing primer prior to sequencing. In this chapter, we will provide an overview of the analysis of repetitive element DNA methylation by bisulfite pyrosequencing, and we will describe such a protocol that is routinely used in our laboratory.

7.1. Introduction

Transposable elements (TEs) comprise approximately half of the human genome (Lander, Linton et al. 2001). Among TEs, the long interspersed elements (LINEs) and short interspersed elements (SINEs) are most common and well-studied. The most common SINEs in mammals are the *Alu* elements. LINE-1 and *Alu* methylation can be altered in response to stress, infection, diseases (Byun, Motta et al. 2013), and they are linked with the genomic instability that is implicated in genetic disorders such as gastrointestinal stromal tumors, myeloma, ependymomas and lung cancer (Su, Shao et al. 2012). The methylation of these elements reduces genomic instability by controlling LINE-1 and *Alu* induced retrotransposition. Different classes of TEs, such as retrotransposons and DNA transposons, display different susceptibility to changes in DNA methylation in response to environmental exposures or disease (Choi, Worswick et al. 2009, Guo, Byun et al. 2014). Furthermore, different subfamilies of LINE-1 and *Alu* elements, generated through mutations over the evolutionary history of the elements, show differential sensitivity to exposome (Byun, Motta et al. 2013).

Bisulfite pyrosequencing is a technique based upon the "sequencing by synthesis" approach. It provides quantitative and highly reproducible methylation data at single-base resolution, and it requires relatively low quantities of DNA (Florea 2013). Pyrosequencing assays for the analysis of TE (LINE or *Alu*) DNA methylation interrogate several thousand copies of elements across the genome, as opposed to one unique locus. CpG sites are particularly enriched at TEs, accounting for >65% of those found throughout the human genome (Lai, Chen et al. 2014), and therefore the methylation of these elements has been suggested as an indicator of global DNA methylation (Yang, Estecio et al. 2004, Baccarelli, Wright et al. 2010, Zhu, Hou et al. 2012). However, several investigations have reported poor correlations in normal tissues between the DNA methylation of TEs and global genomic DNA methylation, as determined by the 'gold standard' of high performance liquid chromatography (HPLC) (Wagner and Capesius 1981, Feinberg and Vogelstein 1983). Nonetheless, LINE-1 and *Alu* methylation can serve as a robust surrogate marker of global DNA methylation for some purposes, such as in cancer cells, where profound TE

demethylation contributes to a global loss of nuclear DNA methylation (Baba, Huttenhower et al. 2010, Lisanti, Omar et al. 2013).

Here, we describe a protocol routinely used in our lab for the analysis of DNA methylation of TE.

7.2.Materials

- Human genomic DNA: up to 1.0 µg of DNA is required.
- Bisulfite-converted DNA: this should be prepared using 0.5 - 1.0 µg of isolated genomic DNA in conjunction with a commercially available bisulfite conversion kit. The eluted bisulfite-converted DNA (20 - 40 µl) can be aliquoted and stored at −80 °C until required for use.
- LINE-1 PCR primer sets (100 µM): for the PCR-based amplification of LINE-1 subfamilies from bisulfite-converted DNA and the performance of bisulfite pyrosequencing (See Note 1 and Table 1).
- Pyrosequencer and Vacuum Workstation: e.g. PyroMark Q96 MD system (QIAGEN, Valencia, CA).
- PyroMark Gold Q96 Reagents: enzymes, substrate and nucleotides required to perform the pyrosequencing reaction (QIAGEN, Valencia, CA).
- PyroMark Binding Buffer, Annealing Buffer, Denaturation Solution and Wash buffer (QIAGEN, Valencia, CA).

Table 1: Bisulfite pyrosequencing assays for LINE-1 subfamilies.

LINE-1 subfamilies	Forward primer	Reverse biotinylated primer	Sequencing primer	Sequence to analyze	Annealing temperature (°C)
L1 P A5	TTAGTTAA GGGAAGA GGGGATA AA	ATAAACAT AAAACCCT CTAAACCA AACA	TTAGTTA AGGAAG A	GGGGATAAA C/TGGTATTT GGAAAATC	40
L1 P A2	TTAGATAG TGGGYGT AGGTTAGT GGGT	CCTCCRAA CCAAATAT AAAATAT AATCT	GAGTTAA AGAAAGG G	GTGAC/TGG AC/TGTATTT GGAAAATC/ TGGGTTATT TTTATT	55
L 1 H s	TTTTGAGT TAGGTGT GGGATAT A	AAAATCA AAAAATTC C CTTTC	AGTTAG GTGTGG ATATAG T	TTC/TGTGG TGC/TGTC/T GTTTTTTAA GTC/TGGTT TGAAAAGC/ TGTA	56.3
L1Ta	GGGTTAG GGAGTTTT TTTTT	CTCTAAAC CAAATATA AAATATA	GGGTTAG GGAGTTT TT TTTT	C/TGAGTTA AAGAAAGG GGTGAC/TG GAC/TGTATT TGGAAAATC /TGGGTTATT TTTAT	55

7.3.Method

7.3.1. PCR amplification of bisulfite-converted DNA

PCR amplification can be performed using 10 - 20 ng of bisulfite-converted DNA, and the following thermocycling conditions: 15 minutes at 95 °C; 45 cycles of (94 °C for 30 seconds; annealing temperature (Table 1) for 30 seconds; 72 °C for 30 seconds); 72 °C for 5 minutes. The specificity and yield of PCR products should be checked for each reaction by visualization on an agarose gel.

7.3.2. Bisulfite pyrosequencing

7.3.2.1. Sample preparation

- Prepare the PyroMark Binding Buffer mixture as follows; PyroMark Binding Buffer: 38 µl, streptavidin-sepharose beads: 2µl, dH_2O: 30 µl.
- Dispense 70 µl of Binding Buffer mixture into each well of a 96-well plate and add 10 µl of PCR product.
- After sealing the plate with an adhesive cover, incubate the plate for 10 minutes with vigorous shaking.
- Prepare the Annealing Buffer mixture as follows; PyroMark annealing buffer: 11.64 µl, sequencing primer: 0.36 µl.
- Dispense 12 µl of Annealing Buffer mixture into a PyroMark Q96 HS Plate (QIAGEN, Valencia, CA).

7.3.2.2. Strand separation

- Fill the Vacuum Workstation with the corresponding buffers (~180 ml of 70 % ethanol, Denaturation Solution, Wash Buffer, and Milli-Q grade water).
- Apply vacuum to the vacuum prep tool.
- Prime the probes of the vacuum prep tool by immersion in the Milli-Q water for approximately 20 seconds.
- Capture the beads containing the immobilized PCR templates on the filter-probes by slowly lowering the vacuum prep tool in to the 96-well PCR plate from Step #3.
- Place the vacuum prep tool into 70 % ethanol and let the solution flush through the filters for 5 seconds.
- Place the vacuum prep tool into the Denaturation Solution and flush it through the filters for 5 seconds.
- Place the vacuum prep tool into the Wash Buffer and flush it through the filters for 5 seconds.
- Switch off the vacuum using the switch on the vacuum prep tool handle and completely remove it from the vacuum station.

- Release the beads in a PyroMark Q96 HS Plate filled with the annealing buffer mixture by gently shaking the vacuum prep tool.
- Seal the PyroMark Q96 HS Plate with an adhesive cover.
-

7.3.2.3. Primer annealing

- Heat the PyroMark Q96 HS Plate at 80 °C for 2 minutes using the PSQ HS 96 sample prep thermo plate kit.
- Remove the plate from the heating block and leave the samples on the bench for 10 minutes in order to cool to room temperature.

7.3.2.4. Pyrosequencing reaction

- Load the pyrosequencing reagents into the cartridges. The volumes required for each reagent are automatically calculated and described within the PyroMark CpG software.
- After performing the dispensing tip test, run the assay within the PyroMark CpG software. Once the run is completed, the data can be extracted (Figure 1).

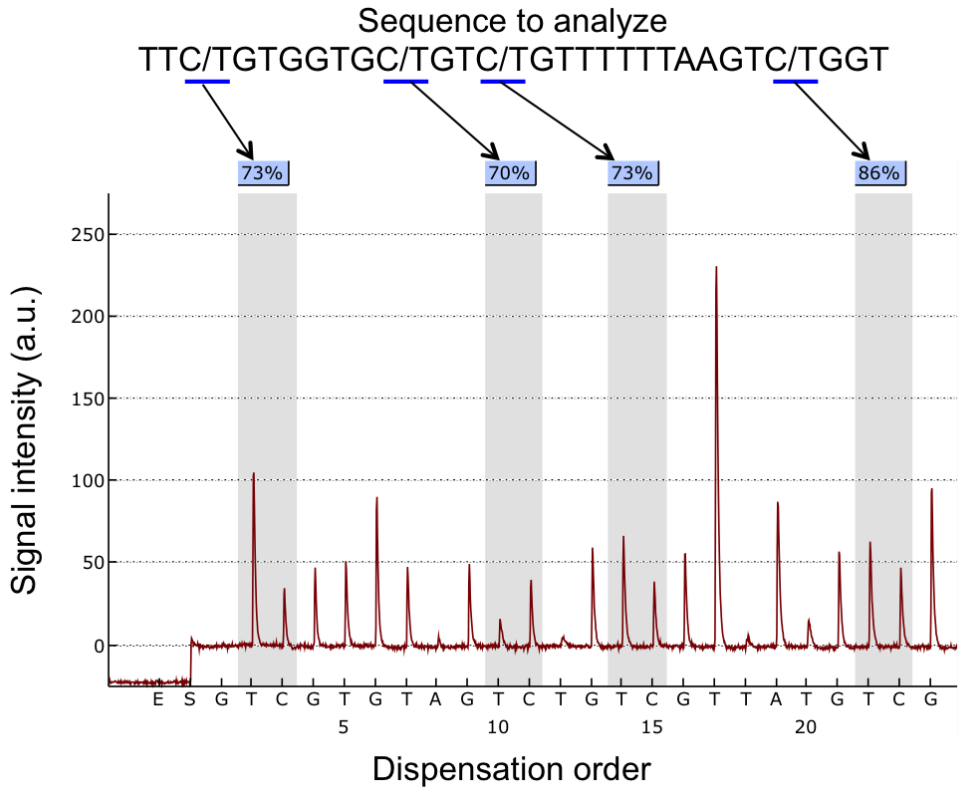

Figure 1: Pyrogram of multiple CpG sites within the LINE-1 sequence. The Y-axis gives intensity (in a.u; arbitrary units), and the X-axis is the nucleotide dispensation order. The LINE-1 sequence analyzed in this assay is given at the top of the figure. Methylation at four CpG sites (highlighted in gray) is quantified in a single pyrosequencing run. Above the gray bars are the percentage methylation levels at each CpG site.

7.4.Notes

- The conventional LINE-1 assay (Yang, Estecio et al. 2004) is based upon the L1HS sequence (Table 1). The 'LINE-1 assay' referred to throughout this chapter is that based upon interrogation of L1HS sequences.
- Repetitive elements DNA methylation in other species, such as mice, rat and monkey, can be measured using bisulfite pyrosequencing. However, the repetitive element sequences are different from those in humans, and therefore the bisulfite pyrosequencing primers have to be carefully designed for each species. These primer sequences are available upon request.
- DNA methylation analysis using bisulfite pyrosequencing is applicable to a wide variety of biological samples, such as DNA isolated from cultured cells, whole blood samples and other body fluids (buccal cells, saliva, nasal swab

samples, etc.), tumor tissue from different sources, and processed samples such as formalin-fixed paraffin embedded tissue samples.

- The PyroMark CpG software has in-built quality control parameters to assess the overall performance of the sequencing run. The software also supports analysis, such as methylation frequency, mean methylation values per well, and replicates and deviation from expected methylation pattern. One important aspect of methylation analysis by pyrosequencing is to assess the overall bisulfite conversion efficiency. Since all non-CpG cytosine residues are converted to uracil and then to thymine in the subsequent PCR reaction, the relative incorporation of thymines and cytosines into the elongated DNA at these sites informs upon the efficiency of the bisulfite conversion reaction.
- Appropriate quality controls are required at each stage of the experiment. Quality control samples should be run alongside the samples of interest for the bisulfite-conversion of DNA, the PCR reaction, and the pyrosequencing run.

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8. Chapter 8

8.1. Concluding discussion

8.1.1. Methods to study environment induced methylation changes

DNA methylation is one the most studied epigenetic modifications. DNA methylation is involved in regulation of pathways of cellular haemostasis. Exposure of environment agents has the potential to alter the cells DNA methylation machinery. Cells with altered methylation patterns can initiate the process of carcinogenesis (Brookes and Shi, 2014). It is therefore important to understand the DNA methylation changes induced by exposure to environmental agents. In current research, methods were developed to quantify the DNA methylation changes induced by exposure to environmental stressors.

8.1.1.1. Global DNA methylation and hydroxymethylation

In order to understand the effects of environmental exposure on cellular epigenetics, it is required to investigate the distribution of epigenetic modifications in the genome. Different methodologies have been employed in order to investigate the tissue distribution of cellular epigenetic modifications e.g., global DNA methylation and hydroxymethylation. Methods have been developed to determine the DNA methylation profiles at the global (% 5mC in the genome) or gene-specific (locus-specific) level (Zuo et al., 2009). In the current project, methods for both global DNA methylation and hydroxymethylation were developed and validated. Methods for quantification of global 5mC and 5hmC were developed on LC-MS/MS platform. Methods validation was performed in a pilot study where healthy humans were requested to participate in the study (chapter 6). Global 5mC and 5hmC levels were quantified in blood and saliva DNA of these individuals. The amount of 5mC in the genome quantified by our method was described to be similar to the ones reported by other studies (Dwi Putra et al., 2014, Thompson et al., 2013). %5mC quantified by the current method is in agreement with the previous studies where median 5mC contents of 4.1% were observed (Kok et al., 2007). This proved the global 5mC analysis developed in-house to be in line with other studies profiling 5mC levels.

Global 5hmC quantification recently gained great interest as it represents an intermediate pathway of DNA demethylation (Pastor et al., 2013). Little is known about the tissue distribution of global 5hmC in different species. In order to investigate the 5hmC amount in mice lung tissue, we developed and validated the LC-MS/MS based method for the 5hmC quantification (chapter 6). In literature, data is scant on the tissue distribution of the 5hmC amount for different species. Here, we report lower global 5hmC contents compared to the other studies reporting global DNA 5hmC (Le et al., 2011). In essence, in-house developed LC-MS/MS method allowed rapid and simultaneous quantification of both 5mC and 5hmC in a single run. This method was employed to quantify the global 5mC and 5hmC levels in vitro and in humans blood DNA samples exposed to chemicals. The amounts of 5mC and 5hmC quantified by this method, in vitro and in human blood DNA samples under different chemicals-exposure settings are discussed below.

8.1.1.2. Gene specific methylation

For gene specific methylation analysis, different methods and techniques are available to investigate the CpG specific methylation marks. For example, methyl-specific PCR, methyLight technique, pyrosequencing, microarrays and whole genome sequencing based assays for methylation profiling (Plongthongkum et al., 2014) These methodologies differ, for example, in terms of sample requirement, batch throughput, CpG coverage. In the current study, we used PCR-pyrosequencing to investigate methylation status of multiple CpG sites at the promoter regions of genes. Bisulfite-treated PCR pyrosequencing is regarded as the "gold-standard technology" as it enables mapping methylated sites at single base pair resolution (Tost and Gut, 2007).

In current research, PCR-pyrosequencing assays were originally developed for human LINE-1 and Alu sequences, and were validated in a pilot study where healthy humans were recruited to investigate the LINE-1 and Alu methylation status in their blood and saliva DNA. The LINE-1 and Alu methylation levels reported in the current research are in line with the published methylation values of human LINE-1 and Alu sequences (Byun et al., 2013). However, we observed lower methylation levels for

LINE1 and Alu elements in saliva DNA compared to their methylation levels in blood DNA. This was expected since saliva comprises mainly the dead cells, which have altered methylation patterns as compared to the blood cells where the methylation levels are tightly controlled. Further, PCR-pyrosequencing assays were developed for nineteen mouse genes to investigate their gene promoter methylation in mice exposed to nanomaterial. Validation of these PCR-pyrosequencing assays were done on standard mouse DNA samples (samples obtained by pooling 5 ng of DNA from each of randomly selected 10 mouse lung DNA samples).

8.1.2. DNA methylation changes after exposure to chemicals

Research conducted in the current project was aimed at delineating the complex interplay between environmental stressors and epigenetics. It is known that epigenetic alterations lead to disease process e.g., carcinogenesis, but what is not fully known is how environmental exposure leads to epigenetic alterations. Here, we filled this knowledge gap by investigating the hypothesis that; exposure to chemicals and nanomaterial could induce epigenetic changes in vitro, in animals and in humans.

8.1.2.1. Exposure to organic solvents, hydrocarbons, halocarbons, cytostatic agents in vitro

We hypothesized that exposure of organic solvents, halocarbons, polyaromatic hydrocarbons, cytostatic agents, to cells will induce global DNA methylation alterations in vitro (chapter 2). Different classes of chemicals used in the in vitro study are known to induce DNA damage i.e., DNA strand breaks, DNA adducts; which might lead to carcinogenesis. However, recently it is proposed that carcinogenesis induced by exposure of chemicals, traditionally considered as genotoxic, also follows epigenetic pathways (Pogribny et al., 2010). Research conducted in the current project corroborated that DNA methylation changes are an early event in response to exposure of environmental stressors. As shown in chapter 3, global DNA hypomethylation was observed in response to exposure of benzene and its metabolite hydroquinone in human cells. However, similar patterns of global DNA

hypomethylation were not observed when human cells were exposed to styrene metabolite. Noticeably, styrene exposure induced global DNA hypomethylation, while its metabolite styrene oxide exposure did not induce global DNA methylation changes in cells. This highlighted the subtle differential sensitivity of cellular epigenome to chemicals with similar mode of actions. Inside the cells, styrene is biotransformed to styrene oxide (Carlson, 2012). It is possible that enzymatic activities render cells epigenetically non-responsive to styrene oxide. This showed that cells DNA methylation is very dynamic and specific in response to chemical exposure.

Similar patterns of global DNA hypomethylation were also observed in human cells exposed to halocarbons e.g., carbon tetrachloride, and trichloroethylene. Mechanistically, DNA hypomethylation effects of halocarbons could be linked with their association with methionine metabolic pathways (Varelamoreiras et al., 1995, Tao et al., 1999). In contrast to above observations, we did not observe DNA methylation changes in vitro in response to PAHs such as benzo[a]pyrene, cyclophosphamide, benzo[a]fluoranthene, benz[a]anthracene and cytostatic agents such as mitomycin C, cyclophosphamide, acrylamide etc. PAHs and cytostatic agents are shown to induce DNA damage in cells (Perera et al., 2005, Recio et al., 2010), however on the DNA methylation endpoint, these agents are not shown to induce global DNA methylation changes in human cells. Although, no global DNA methylation changes were observed under the current settings, it is possible these chemicals can still induce passive DNA methylation changes by diluting methylated cytosine during successive rounds of mitosis. This could not be confirmed in the current settings, since TK6 cells were exposed to these agents for one doubling time. It is also possible that exposure to these agents lead to gene specific methylation changes. Currently, we are investigating such gene specific methylation changes. In essence, these findings showed the induction of global DNA methylation changes as an early response of chemical-exposure in cells.

Chemical induced global DNA hypomethylation observed in TK6 cells could poise cells to carcinogenesis. Similar to methylation changes observed in TK6 cells, cancer cells also frequently exhibit global loss of DNA methylation. In cancer cells, global DNA hypomethylation is reported to occur early during carcinogenesis, and this loss

of methylation could potentiate the carcinogenesis by activating the oncogenes and cell survival (Ehrlich, 2009). This association of global DNA hypomethylation with the early stages of carcinogens could be used as cancer biomarker. Tissue specimens obtained from suspected individuals could be screened for the global loss of methylation in order to calculate the risk of developing cancer.

8.1.2.2. Exposure to organic solvents in humans

DNA methylation alterations induced by organic solvents in vitro were further confirmed in human population who had previous exposure of organic solvents. Humans chronically exposed to solvents are at risk of developing various diseases like cancer and neurobehavioral disorders. In current study, DNA methylation patterns associated with exposure to organic solvents were investigated, which might be used as markers of solvent exposure to predict the disease susceptibility in populations at risk. Global DNA hypermethylation was observed in humans exposed to mixture of solvents e.g., benzene, formaldehyde, chloroform compared to the non-exposed population. Parameters of organic solvents exposure (i.e., total exposure time, cumulative exposure index) were shown to be negatively correlated with global DNA methylation in solvent-exposed individuals. Interestingly, as shown in chapter 4, DNA methylation patterns in CTE patients were comparable to the reference populations. Since CTE patients do not have active exposure to solvents, while the exposed-group were under current solvent exposure at the time of sampling, this could explain the observed effects of DNA hypermethylation in solvent-exposed individuals. These results highlighted the dynamic regulation of DNA methylation changes, and that the DNA methylation changes could have been reversed in the CTE population after the exposure was ceased.

This leads to an important outcome on the “time window” that has to be taken into consideration in order to observe the DNA methylation effects in response to exposure. DNA methylation effects could be diluted/or reversed outside this time window. In previous studies, both sequence specific DNA hypo- and hypermethylation response is reported in peripheral blood DNA after exposure with low levels of benzene (Bollati et al., 2007). Also in the context of cancer, sequence

specific hypermethylation while on global scale of DNA hypomethylation is observed which drives epigenetic carcinogenesis (Rountree et al., 2001). These observations lead to conclude that apparently contradictory results, induced by solvents exposure under different systems, can hold physiological relevance.

Recent in vitro studies also showed that solvent exposure induces DNA methylation changes (Liu et al., 2011). Mechanistically, how exposure to solvents leads to DNA methylation alterations is largely unknown. It is suggested that oxidative stress could be intermediary pathway leading to solvents induced epigenetic changes (Dreiem et al., 2005). Another important observation drawn from this study was gene-environment interaction that could define individuals' disease susceptibility on exposure to solvents. Gene-environment interaction between *GSTP1* and total exposure time for global DNA hypomethylation were observed. This is in line with the findings that genotypic polymorphism in drug metabolizing enzymes affects disease susceptibility (Godderis et al., 2010).

On comparison with in vitro findings, where solvent exposure induced global DNA hypomethylation, findings in humans exposed to solvents showed results contrary to the in vitro results. Global DNA hypomethylation was observed in cells exposed to chemicals in vitro. While global DNA hypermethylation with dose- and time-dependent hypomethylation was observed in humans exposed to chemicals. On one sight, these apparently contradictory findings depict the complexity of translating in vitro findings in humans. On other sight, these observations highlight the importance of exposure period and exposure dose in studying cells epigenetic response to xenobiotic. DNA methylation is considered reversible and dynamic epigenetic modification (Ramchandani et al., 1999). In one study, DNA methylation changes were reported to take place as early as 20 minutes following the stimulation of T-lymphocytes in vitro (Bruniquel and Schwartz, 2003). On the note of dynamicity of DNA methylation marks, short term in vitro exposure of cells to arsenic was shown to induce pronounced loss of global DNA methylation. While long term in vitro exposure of cells to arsenic was associated with slight hypomethylation of LINE-1 compared to the greater degree of LINE-1 hypomethylation on short term in vitro exposure to arsenic. These observations highlight the importance of time-window in studying the epigenetic response to environmental stressors. In this study, chemical

exposure in TK6 cells was studied for 24 hours. TK6 cells responded to the chemical exposure with global loss of DNA methylation. But, if TK6 cells were exposed to even longer time periods (e.g., 48 hours or even longer periods), it could be expected that; cells either respond by further demethylating the genome, or cells adaptive mechanism stops further demethylation of the genome. In these demethylation scenarios, it is difficult to conclude the time-point at which cells physiological epigenetic response turns into pathological epigenetic response after exposure to environmental stressors. Global loss of methylation could potentially lead cells to genome instability i.e., a hallmark of cancer; thus leading towards epigenetic carcinogenesis. The time-window to initiate the epigenetic carcinogenesis after cells exposure to chemicals could be different for different chemicals. Hence, it is required to characterize the time-window for the cells to initiate pathological epigenetic response after exposure to different environmental stressor. Global DNA methylation changes observed in vitro and in humans in response to chemical exposure could potentially be linked with the initiation of a disease process. Such DNA methylation alterations have been described to take place e.g., in cancer, cardiovascular disorders, neurological disorders (Brookes and Shi, 2014, Webster et al., 2013, Babenko et al., 2012).

Another important aspect of DNA methylation patterns observed in CTE patients is the phenomenon of reverse causality. It is important to establish if the occurrence of epigenetic changes is due to the exposure or is it secondary to the emergence of CTE itself; and which lies on the casual pathway linking the two (i.e., linking exposure/or disease process to observed epigenetic changes). Without establishing the reverse causality, it is challenging to prove if the observed epigenetic changes are linked with exposure. In the current study, it is difficult to establish if the observed global methylation profiles of CTE patients is under the influence of longer exposure time or the observed methylation patterns are due to the CTE itself.

8.1.3. Exposure to nanomaterial

We investigated the epigenetic alterations associated with exposure to nanomaterial. Humans have been subject to nanomaterial exposure through various sources, but it

has been recent with the large-scale production of engineered nanoparticles that their exposure triggered scientific community to investigate adverse health effects associated with nanomaterial exposure. Exposure to nanomaterials has the potential to alter the cellular epigenome. However, currently no investigations have been done in this direction to assess the epigenetic alterations associated with exposure to nanomaterial. Here, we filled this knowledge gap by investigating the hypothesis that; cellular exposure to nanomaterial would induce epigenetic changes in animals. In order to test the hypothesis, we developed and validated the techniques for the quantification of global DNA methylation and hydroxymethylation in the genome, as described in the previous section. We developed and validated methods to investigate the CpG methylation at promoter regions of genes. Development and validation of the CpG specific promoter methylation methods were discussed above.

8.1.3.1. Exposure to gold nanoparticles and carbon nanotubes in mice

In the current project, it is shown for the first time that exposure to nanomaterial leads to gene promoter methylation alterations in animals (chapter 5). Exposure of gold nanoparticles leads to the induction of both hypomethylation and hypermethylation in the promoter CpGs in exposed animals. For example, promoter CpGs hypermethylation was observed for *Atm*, *Cdk* and *Gsr* genes; while promoter CpGs hypomethylation was observed in *Gpx* gene. Genes with altered promoter methylation were shown to take part in divers cellular process e.g., cell cycle process, apoptotic process, immune system process, metabolic process and response to stimulus pathways. Functionally gold nanoparticles induced promoter hypermethylation in *Atm* and *Cdk* genes is important. These genes are important regulator of cell cycle, and hypermethylation of these genes could affect the normal functions of these genes in controlling the cell cycle events.

These findings raised important questions on the biocompatibility of engineered nanoparticles. Of particular note were the gold nanoparticles of 60 nm diameter. Generally, it is expected that cells with exposure of smaller nanoparticles (such as 5 nm gold nanoparticles) will manifest higher biological activity compared to the larger

nanoparticles. Contrary to it, current findings reported more gene promoter methylation perturbations induced by exposure to gold nanoparticles of 60 nm in diameter compared to gold nanoparticle of 5 nm diameter. This could partially be explained by the physicochemical features of gold nanoparticle used in the current research. Gold nanoparticles were citrate coated. Gold nanoparticle of 5 nm diameter have higher surface area for the attachment of citrate compared to gold nanoparticles of 60 nm and 250 nm diameters. High contents of citrate on the surface of gold nanoparticles of 5 nm diameter could potentially mask the effects of these nanoparticles on gene promoter methylation. These 5 nm gold nanoparticles also have higher tendency to agglomerate in the biological media compared to the gold nanoparticles of 60 and 250 nm diameters, as shown in chapter 5, this could also lead to suppressed biological activity.

In nanomaterial-exposed mice with significant promoter methylation changes, no significant DNA damage was observed. This observation, that nanomaterial exposure can induce epigenetic changes in the genome without DNA damage, implicated epigenetics as an early response to exposure; instead of mice adaptive mechanism or secondary response to genetic damage. The above findings are preliminary that warrant further investigations with different model systems and more types of nanomaterial to be included. In contrast to gold nanoparticle, exposure to carbon nanotubes (SWCNTs, MECNTs) did not induce major gene promoter methylation alterations in mice. Carbon nanotubes exposure affected the *Atm* gene promoter methylation. This is in contrast to other reports where carbon nanotubes were described more toxic and even carcinogenic. Carbon nanotubes exposure was shown to induce effects similar to asbestoses exposure (Pacurari et al., 2010). On the global DNA methylation and hydroxymethylation endpoints, carbon nanotubes were shown to have no effect in mice lung DNA. No other research groups have published epigenetic effects of carbon nanotubes in animal models or in humans, and this makes it difficult to compare the current findings with others.

Recent epidemiological studies have linked exposure of different metals and particular matter in humans to the altered epigenetics patterns, and proposed that cells with altered methylation marks could act as pre-neoplastic cells (Weidman et al., 2007, Libbrecht et al., 2005). In current report, nanomaterial are also shown to have

potential to alter the cells epigenetic patterns. The magnitude of these epigenetic alterations to render cells to neoplastic phenotype has yet to be investigated. So it is important to further characterize nanomaterial induced epigenetic changes, and to limit the environmental release of nanomaterial owing to their potential adverse biological effects in cells.

8.1.4. Conclusion

The major epigenetic mechanisms comprise the DNA methylation, histone PTMs and mciroRNAs. DNA methylation changes in response to chemical and nanomaterial were investigated in current report. Alterations in histone PTMs and in microRNAs were not investigated in this report. It is, however, expected that chemical and nanomaterial could also affect the histone PTMs and microRNAs. Number of studies has described the alterations in histone PTMs and in mciroRNAs in response to environmental stressors. Environmental stressor induced histone PTMs changes are suggested to drive carcinogenesis (Martinez et al., 2011, Dik et al., 2012). Regarding to microRNAs; exposure to heavy metals i.e., cadmium, arsenic, aluminium leads to alterations in the expression levels of microRNAs (miR-146a, miRNA-19a, miR-9 respectively). Exposure to cigarette smoke is also shown to alter the expression profile of microRNAs (Hou et al., 2011). Altered expression levels of microRNAs are suggested to drive the process of carcinogenesis. In fact, microRNAs are suggested to play role similar to tumour suppressor genes and oncogenes (Garzon et al., 2009). Thus, all major epigenetic mechanisms i.e., DNA methylation, histone PTMs and mciroRNAs are susceptible to alterations in response to environmental exposure. Altered epigenetic patterns in cells can further lead to carcinogenesis and other multifactorial diseases.

Concluding the main findings from this research, it can be reported that cells can initiate epigenetic changes in response to a variety of environmental stressors. Chemicals and nanomaterial represent different categories of environmental stressors. This shows that the activation of epigenetic mechanisms is common in response to diverse types of environmental stressors. We can report that epigenetic modifications can be considered as early makers of xenobiotic stress, which could confer cells

neoplastic phenotype. However, the extent and magnitude of epigenetic stress required for cells to transform into neoplasm needs to be further investigated. It is also important to note that gene promoter methylation at single CpG site can functionally act similar to single nucleotide polymorphism (SNP). In this context, where a single SNP or mutation can alter disease susceptibility, methylation at single CpG sites can also affect the binding of transcription factors and thus regulates the gene expression. Current findings elaborated this phenomenon, where methylation alterations are reported at single CpG sites but surrounding CpG sites remained unaffected. These findings will further help in delineating cellular signalling mechanisms driving these epigenetics alterations. Findings in this report will also enable scientists to build strategies of effective biomonitoring for human population at risk, and to develop early epigenetic marker of disease susceptibility.

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9. Chapter 9

9.1.Summary of current research

9.1.1. Background

Humans are exposed to a variety of environmental stressors during their life time e.g., organic solvents, polyaromatic hydrocarbons, halocarbons, cytostatic agents. Due to anthropogenic factors, new environmental stressors have been emerged e.g., engineered nanoparticle (NPs), and nanotubes. NPs are defined as particles with at least one particulate dimension less than 100 nm. Exposure to environmental stressors has shown to induce adverse health effects. Recent studies also raised concerns about effects in cells in response to nanomaterial exposure. So far, it remains difficult to assess the impact of environment on health. Therefore, it is important to get insight in the disease mechanisms.

It is proposed that exposure to environmental stressors can lead to epigenetic changes similar as observed in diseased cells, e.g. cancer cells. Epigenetics encompasses structural modifications of DNA and histones, and microRNAs which regulate the gene expression without affecting the DNA sequence. Methylation of 5^{th}-carbon of cytosine bases in DNA (5mC) is one of the most studied epigenetic modifications. 5mC modification can also be demethylated in cells by the actions of different enzymes leading to the production of an intermediate modification called DNA hydroxylmethylation (5hmC). We hypothesize that exposure to environmental stressors could alter the DNA methylation marks globally or in gene specific manner.

9.1.2. Materials & methods

In the current project, global DNA methylation and hydroxymethylation and gene promoter methylation changes associated in response to exposure of a range of chemicals and nanomaterial (gold NPs (AuNPs) of three size, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs)) are investigated in the laboratory settings and in humans. Global 5mC and 5hmC was quantified by liquid chromatography- mass spectrometry (LC-MS/MS) method, while

gene promoter methylation was quantified by bisulfite-PCR pyrosequencing of 17 genes selected from different pathways commonly affected by exposure to xenobiotic.

9.1.3. Results & discussion

In the laboratory settings, global DNA hypomethylation was observed in TK6 cells in response to exposure of benzene (p=0.0295), hydroquinone (p=0.0108), styrene (p=0.0115), carbon tetrachloride (p=0.0475) and trichloroethylene (p=0.0294). In studying the effects of solvent exposure in humans, DNA hypermethylation (p = 0.001) was observed in in blood DNA of individuals working with solvents compared to the control group. In exposed individuals, *GSTP1* genotypic polymorphism was found to be significantly associated (p=0.033) with global DNA hypomethylation, which describes the potential gene-environment interaction in the aetiology of solvent-induced phenotypes.

While studying nanomaterial exposure effects in mice, no global 5mC or 5hmC changes were observed in response to AuNPs, SWCNTs, and MWCNTs exposure in mice lung DNA samples. In mice lung DNA samples, following promoter methylation changes were observed; AuNPs 60nm exposure induced promoter CpGs hypermethylation in *Atm*, *Cdk* and *Gsr* genes, while promoter CpGs hypomethylation in *Gpx* gene; changes in promoter methylation of *Gsr* and *Trp53* was also observed between low and high dose (i.e., dose effect) of AuNPs 60 nm and AuNPs 250 nm respectively; AuNPs size effects on promoter methylation was observed for *Trp53* gene; CNTs exposure affected the promoter methylation of *Atm* gene. In mice blood DNA samples, the only effect occurred was the induction of promoter hypermethylation in *Pparg* gene by exposure of AuNPs 60 nm high dose compared to the AuNPs 60 nm low dose. Epigenetically altered genes were involved in DNA apoptotic process, immune system process, metabolic process and response to stimulus pathways. These finding could implicate the epigenetic pathways in carcinogenesis.

9.1.4. Conclusion

Concluding the major findings, we can report that cells epigenetic alterations are considered as an early event in response to exposure of a verity of environmental stressors. We delineated for a wide variety of chemicals i.e., organic solvents, hydrocarbons, halocarbons, cytostatic; that their exposure in cells leads to alterations in cells global DNA methylation patterns. We also showed that cells exposure to nanomaterial alters methylation changes in gene prompter CpGs. Further, it remains to be characterized the timing at which cells epigenetic response to environmental stressors leads to pathological signalling events which initiate the disease process. Future work should also address; the molecular events that are intermediary between the exposures of environmental stressors to the observed epigenetic changes; and to develop preventative epigenetic screening strategies for human population who are at risk of developing environmental exposure induced malignancies.